A STUDY OF RAT OLFACTORY MECHANISMS

by

Rajinder Kaur Aujla, B.Sc., P.G.C.E.

A thesis submitted for the degree of Doctor of Philosophy

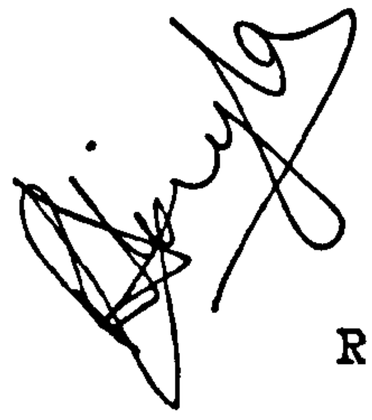
at the

UNIVERSITY OF WARWICK

April 1988

MEMORANDUM

This dissertation is submitted to the University of Warwick in support of my application for admission to the degree of Doctor of Philosophy. It contains an account of my work carried out at the Department of Chemistry under the supervision of Dr. G. H. Dodd. No part of it has been used previously in a degree thesis to this or any other university. The work described is the result of my own independent research except where specifically acknowledged in the text.



R. K. Aujla

1988

Department of Chemistry,

University of Warwick,

Coventry CV4 7AL,

U.K.

ACKNOWLEDGEMENTS

I should like to thank my supervisor Dr. G.H. Dodd for his advice throughout this project. I should also like to thank my colleagues in the Warwick Olfaction Research Group, especially Steve Shirley and Jane Robinson, for their help and useful discussions. I am most grateful to Peter Evans for his valuable time and effort devoted to the GC/MS analyses reported in this thesis.

My thanks are extended to Gallaher Ltd. for the SERC CASE studentship.

Finally, my special thanks are due to my husband, Rajinder, for his moral support and dedicated help in the preparation of the final draft.

To my parents

Sardar and Sardarni Manjit Singh

TABLE OF CONTENTS

ABSTRACT

Membranes prepared from the olfactory mucosa of the rat show a high level of adenylate cyclase activity, typically 500 pmol/min/mg protein. The activity increases in the presence of odorants and is inhibited by calcium. Linkage of receptor to cyclase seems to be mediated by a G-protein. The existence of an odorant-stimulated adenylate cyclase in the rat is discussed. The olfactory tissue is found to contain high levels of intracellular cyclic AMP (ca. 40 pmoles/mg protein), almost twice that found in the brain tissue. Odorants appear to have no effect on these levels. The role of lipids in olfaction is investigated. The olfactory epithelium is found to be a rich source of phospholipids, comprising approximately 80% of the total lipids. The phospholipids identified are PC, PE, PS, SM, PA, PI, PIP, PIP_2, accounting for approximately 41, 23, 9, 11, 4, 10, 2, and 2%, respectively, of the total phospholipids. ^{32}P orthophosphate is incorporated into all the phospholipids; PIP and PIP_2 show the highest rate of incorporation. Attempts are made to investigate the effect of odorants on this rate of incorporation. The fatty acid profile of the rat olfactory phospholipids is found not to be unique to the olfactory tissue. Elaidic acid 81:1t [n-9], however, seems to be present in higher concentrations than observed in other tissues. The fatty acids identified are 14:0, 15:0, 16:0, 16:1[n-9], 17:0, 18:0, 18:1c[n-9], 18:1t [n-9], 18:2[n-6], 20:0, 20:3[n-6], 20:4[n-6], 20:5[n-3], 22:0, 22:4[n-6],22:5[n-6],22:5[n-3], 22:6[n-3], 24:0, and 24:1[n-9].

ABBREVIATIONS

ACTH	Adreno-Cortico-Trophic-Hormone
ANSA	1-Amino-2-hydroxy-4-naphthalene
ATP	Adenosine-5'-triphosphate
BHT	2,6-di-tert-butyl 1,4-methyl phenol
CL	Cardiolipin
cpm	Counts per minute
cyclic AMP/cAMP	Cyclic adenosine-3,5-monophosphate
cyclic GMP/cGMP	Cyclic guanosine-3,5-monophosphate
cyclic Ins1,4,5P_3	1,2 (cyclic) 4,5-triphosphate
DAG/DG	Diacylglycerol
EDTA	Ethylene diamine tetra-acetic acid
EGTA	Ethylene glycol-bis-(aminoethyl ether) N,N'- tetra-acetic acid
EOG	Electro-olfactogram
FAME	Fatty acid methyl ester
GC/MS	Gas chromatography/Mass spectroscopy
$GDP_{\beta}S$	Guanosine 5'-O-(2-thiodiphosphate)
GPPNP	Guanosine 5'-(β-γ-imido) triphospate
GTP	Guanosine-5'-triphosphate
$GTP_{\gamma}S$	Guanosine 5'-O-(3-thiotriphosphate)
HPTLC	High performance thin layer chromatography
IBMX	Isobutylmethylxanthine
Ins1,3,4,5P_4	Inositol 1,3,4,5-tetra kisphosphate
Ins 1,4,5P_3	Inositol 1,4,5 trisphosphate
Meth Prep II™	0.2N methanolic solution of [M-trifluro methyl phenyl] trimethyl ammonium hydroxide

MG	Monoacyl glycerol
PA	Phosphatidic acid
PC	Phosphatidyl choline
PE	Phosphatidyl ethanolamine
Pi	Inorganic orthophosphate
Ptd Ins/PI	Phosphatidyl inositol
Ptd Ins4P/PIP	Phosphatidyl inositol-4-monophosphate
Ptd Ins4,5P_2/PIP_2	Phosphatidyl inositol-4,5-bisphosphate
PLC	Phospholipase C
PME	Phosphomonoesterase
POPOP	1,4-di-2-(5-phenyloxazolyl)-benzene
PPO	2,5-diphenylozazole
PS	Phosphatidyl serine
SEM	Scanning electron microscope
SM	Sphingomyelin
TLC	Thin layer chromatography

CHAPTER 1

OLFACTION: A GENERAL REVIEW

1.1 INTRODUCTION

The sense of smell in higher vertebrates is capable of extremely sensitive detection and accurate identification of practically all airborne chemical compounds. While extensive research has been aimed at the anatomy and electrophysiology of this sensory pathway[1-3], relatively little research has been done on its molecular mechanisms. Recent studies suggest that the first step in vertebrate chemoreception involve membrane-receptor proteins that reside on the cilia of sensory neurons in the olfactory epithelium[4-6]. However, the biochemical properties of these putative receptor molecules remain largely unknown.

The olfactory system appears to share many structural and functional attributes with the visual system. In both, sensory reception takes place at ciliary organelles having a large membrane area and containing the molecular apparatus that mediates stimulus-evoked changes of membrane potential. Whereas the structure and function of rhodopsin, the photo-receptor protein of retinal rods, and several enzymes coupled to it are known in detail[7], virtually no equivalent information is available for the olfactory counterparts. It is only in the last few years that <u>in-vitro</u> preparations for investigating chemosensory mechanisms at the molecular level

have become available. Studies on these preparations are yielding insights into the molecular basis of olfaction.

1.2 THE OLFACTORY APPARATUS

1.2.1 General Structure

Vertebrate olfaction is mediated by the olfactory epithelium, the structure of which has been described in detail[8]. It is 100-200 um thick, and it can be seen to lie upon a series of convoluted structures, the turbinates, and on the cribriform plate (see Fig. 1.1).

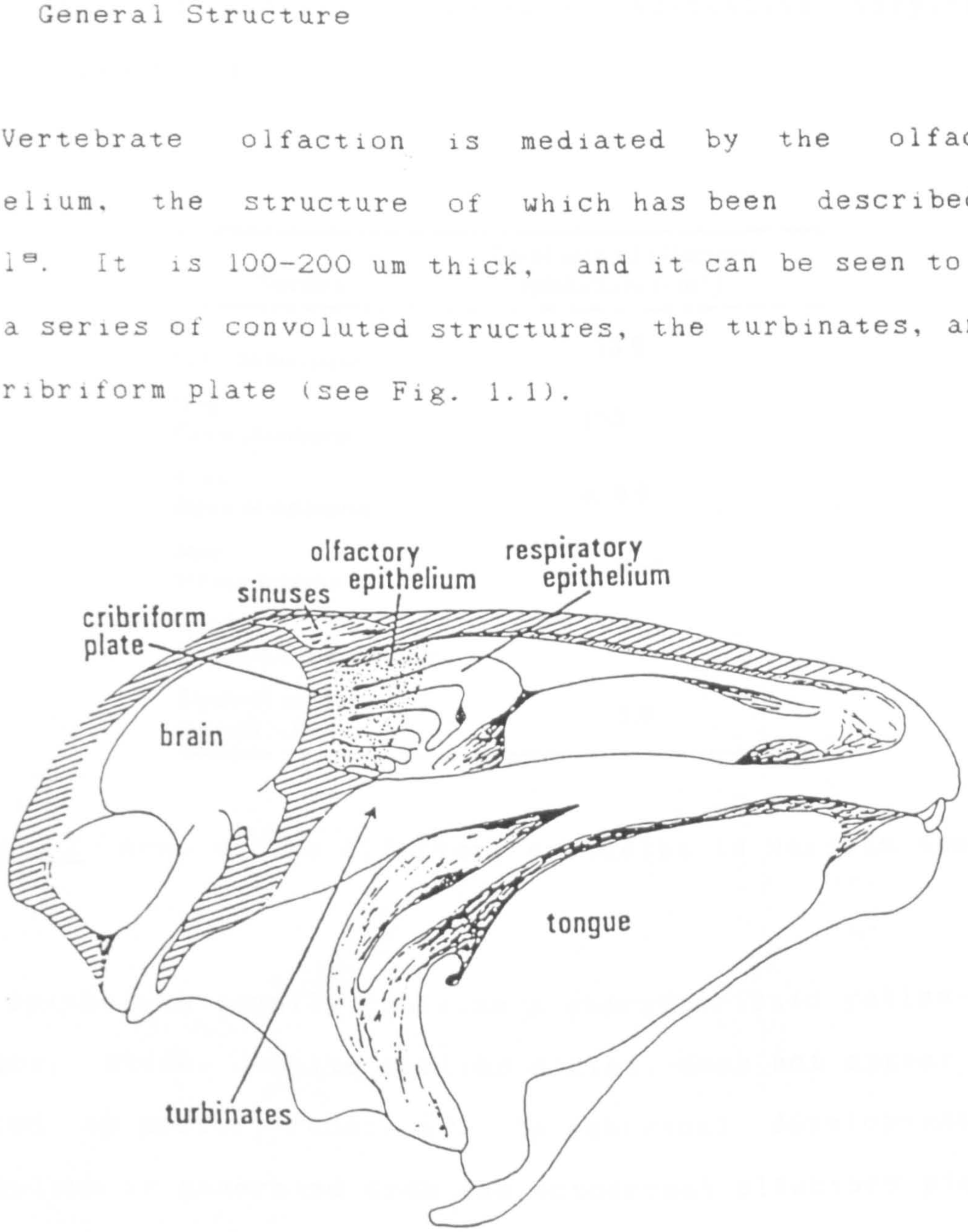


Fig. 1.1 Sagittal section of a sheep's head showing the location of the olfactory region. The nasal septum has been removed. [From Dodd, G.H. and Squirrell, D.J.,(1980) Symp. Zool. Soc. Lond., 45, p37]

It is also found on the posterior part of the septum. The turbinates are rigid to hold opposing faces of the epithelium apart and the system allows a large area of exposed sensory tissue to be packed into a small space. The same basic structure is found throughout the Mammalia with the number of turbinates and the area of olfactory epithelium varying as shown in Table 1.1.

Species	Total area of olfactory epithelium (cm^2)
Cat *Felis domesticus*	13.9
Dog *Canis familiaris*	150
Frog *Rana temporaria*	*c.* 0.5
Man *Homo sapiens*	2—4
Rabbit *Oryctolagus cuniculus*	7.27
Squirrel monkey *Saimiri sciureus*	3.0

Table 1.1 Area of the olfactory epithelia in various species.

The epithelium usually contains a characteristic yellow-brown pigment, which, despite earlier claims, does not appear to be related to sensory function[a]. In embryonal development the epithelium is generated from the ectodermal olfactory placode, separate from other regions of the nervous system[a].

The cell structure of the olfactory epithelium has three

types of cells present with their nuclei arranged in slightly overlapping layers (see Fig. 1.2). The supporting cells are thought to be glia-like in nature and have microvilli on their apical surface. These cells have a role in the secretion of mucus compounds, and they may also function as isolating current sinks, analogous to retinal Müller cells, in guiding the growing sensory neuronal processes and in phagocytosis of shedded dendritic fragments[10].

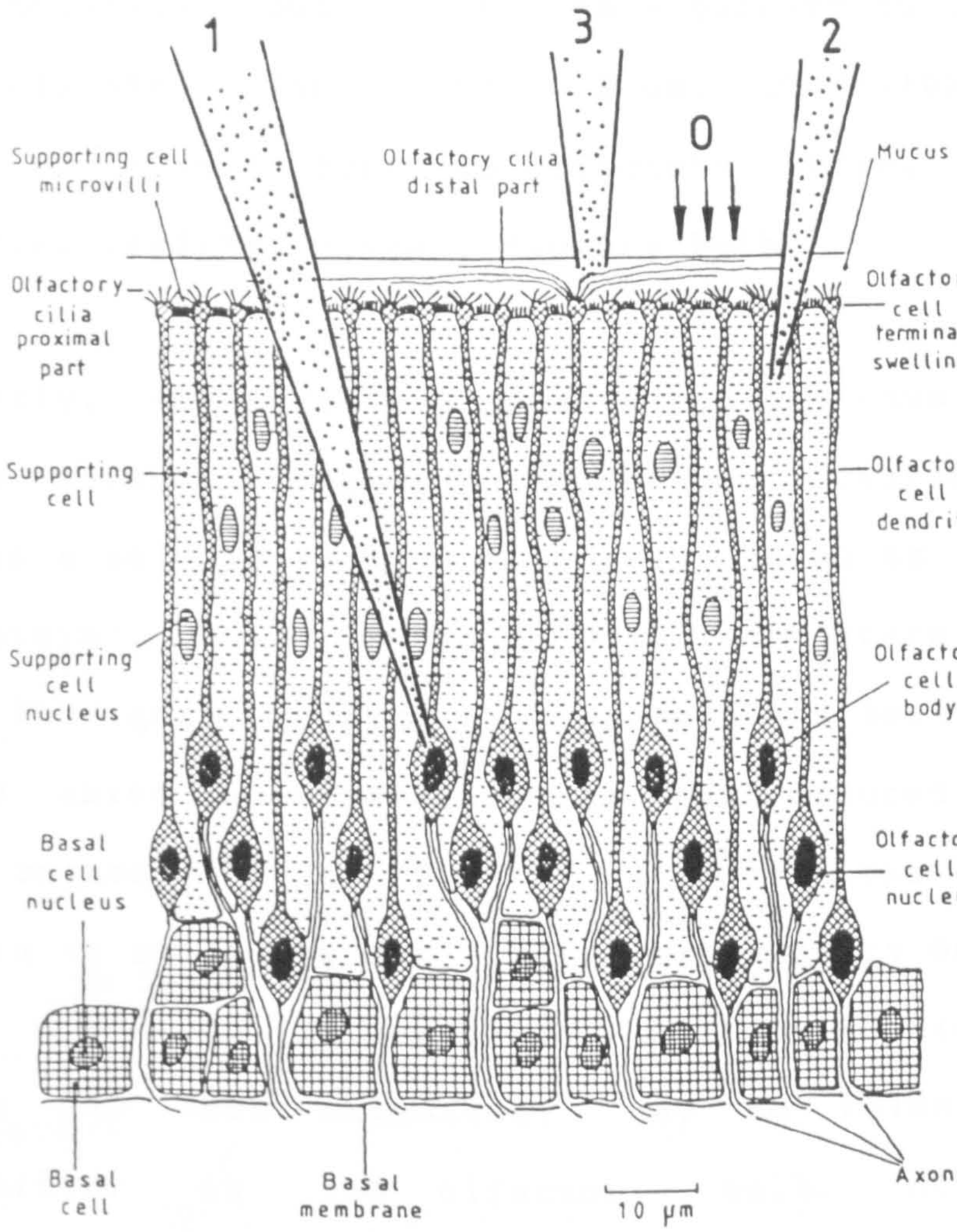


Fig. 1.2 A schematic representation of structure and electrophysiology in olfactory epithelium, showing the positions of electrodes 1, 2, and 3 for Intracellular, Extracellular and Electro-olfactogram recordings, respectively, following stimulation by odorant O.

The sensory neurons are distinctively-shaped bipolar nerve cells, with the cell body located in the middle nuclear layer of the epithelium. Distally, the non-branching dendrite, which has a width of about 2 um, is sent to the surface of the epithelium where it ends in a ciliated terminal swelling, called the olfactory knob, bearing 5-20 long cilia. The terminal swelling protrudes slightly above the level of the surrounding supporting cells. Tight junctions between olfactory dendrites and the supporting cells are impenetrable to charged molecules, but do not form a barrier to lipophylic, uncharged odorants. The axons (0.2 um, unmyelinated) form bundles which join to form the olfactory nerve, the first cranial nerve, leading to the olfactory bulb.

Recently, the olfactory sensory neurons have been shown to possess several intriguing biochemical characteristics. They contain a soluble 18-19 kDa protein, known as 'olfactory-marker protein', that is found only in mature olfactory neurons. The gene encoding this protein has been cloned and its entire amino acid sequence has been deduced from the nucleotide sequence of the cDNA[11]. However, the function of this protein is still unknown. Olfactory sensory neurons also synthesise the dipeptide carnosine (β-alanyl-L-histidine)[12], which, as has been suggested, may be released as a neurotransmitter in the olfactory bulb. However, no postsynaptic effects of carnosine have thus far been described. Olfactory sensory neurons also contain taurine[13], another meurotransmitter candidate, and vimentin[14], a

microfilamentous protein usually associated only with developing but not with mature neurons.

The deepest layer of olfactory epithelium contains basal cells. Considerable evidence suggests that these are stem cells that divide and differentiate, to become functional sensory neurons. This is a rare case of a neuronal population undergoing continuous renewal in adult vertebrates[15]. When the olfactory nerve is severed, the sensory cells degenrate and disappear. Enhanced mitotic activity of the basal cell then gives rise to a newly formed population of sensory cells that send axons and form synaptic connections. A functional epithelium reforms after 30-60 days[16].

1.2.2 Olfactory Cilia

The olfactory receptive apparatus probably resides in the extensions of the sensory dendrites, known as olfactory cilia[17]. These sensory organelles are similar to cilia from other tissues. The proximal portion of the cilia has a diameter of about 0.25 um and a normal 9(2)+2 axonemal structure, but after a few um there is a distinct narrowing of the cilium to a diameter of about 0.15 um and from here on the cilia gradually tapers to about 0.06 um in diameter. The gradual tapering of the distal part of the cilium is accompanied by a progressive reduction in the number of micro-filaments. Thus, any cross-section through the mucus shows cilia with varying numbers of micro-filaments according to the

point at which each has been cut.

Both the number of cilia per neuron and the length of the cilia vary from species to species. For instance, the estimated number of cilia per neuron range from 1-6 for a mole[18], 40-50 for sheep[19], and 100-150 for a dog[20]. The determination of the ciliary length is difficult since the thin sections observed microscopically are unlikely to contain the full length of any cilium and the fragility of the cilia renders them liable to breakage during fixation. Nevertheless, olfactory cilia in the frog have been isolated and found to be unusually long, reaching lengths of upto 200 um[21].

Olfactory cilia are unlike respiratory cilia on the surrounding tissues, which maintain a constant diameter and retain a 9(2)+2 structure of microfilaments throughout their length. The respiratory cilia beat in a synchronous wave-like pattern whereas any motion that the olfactory cilia display is uncoordinated and irregular. Olfactory cilia in some species (including all mammals) are immotile since they lack dinein, the ciliary energy-transducing ATPase[22]. It is therefore unlikely that ciliary motility is important for sensory function. Menco et al.[23] using freeze-fracture techniques have demonstrated in bovines the existence of large numbers of particles in the membranes of the olfactory cilia, compared to corresponding respiratory cilia . It is postulated that these particles might represent olfactory receptor sites.

In being modified ciliary structures, olfactory cilia resemble other sensory organelles, such as rod and cone outer segments in the retina, or hair-like kinocilia in the inner ear[24].

1.2.3 Mucus

A layer of mucus (10-30 um thick) is found overlying the cilia in all classes of vertebrates. Odorants dissolve in this mucus prior to their interaction with the ciliary membrane. The mucus is mobile at the surface, and in man, is composed of over 95% water with salts, mucin, neuraminidase and secretory immunoglulin[25]. It has a pH of about 7.0; the air/mucus partition coefficient for odorants and their diffusion rates through the mucus controls access of odorant molecules to the receptors[26].

The transport of odorants to receptor sites is thought to occur in two sequential steps: (a) the air-borne odorant is transported parallel to the relatively stationary mucoid phase, and (b) the fluid-borne odorant is transported mainly perpendicularly to the surface through the mucus. Mozell and his colleagues[27] have proposed a chromatographic-like model for a differential sorption between air and mucus phases, to account at least partially for odour discrimination, (imposed spatial patterning). Moulton et al.[28] propose that a differential discrimination of receptor sites on receptor neurons of similar responsiveness accounts at least partially

for odour discrimination, (inherent spatial patterning). It is argued that both processes could act in concert to facilitate discrimination amongst odorants. Therefore, the olfactory mucus may play an important role in pre-receptor events in vertebrate olfaction. Fig.1.3 shows the relationships between the air, mucus, cellular compartments, and the corresponding "imposed" & "inherent" spatial patterning hypotheses.

In lower vertebrates such as the frog and the salamander, the height of the olfactory mucus is estimated to be about 35 um^{-1}. The mucus is described as consisting of a superficial water layer estimated to be 5 um high and a deeper, more viscous layer, estimated to be 30 um high. The former is thought to be derived from Bowman's glands and the latter from sustentacular cells. The cilia lie at the interface between the superficial and deep mucus layers. Previous studies have emphasised that free diffusion of molecules through the mucus to the receptor sites plays a dominant role in access of odorants to chemosensitive "active sites"[29]. Thus the height and rheological properties of the mucus layer are major determinants of odorant access. Laboratory and clinical studies have suggested that the volume and rheological properties of mucus secreted varies under a variety of experimental, pathological and therapeutic conditions[30].

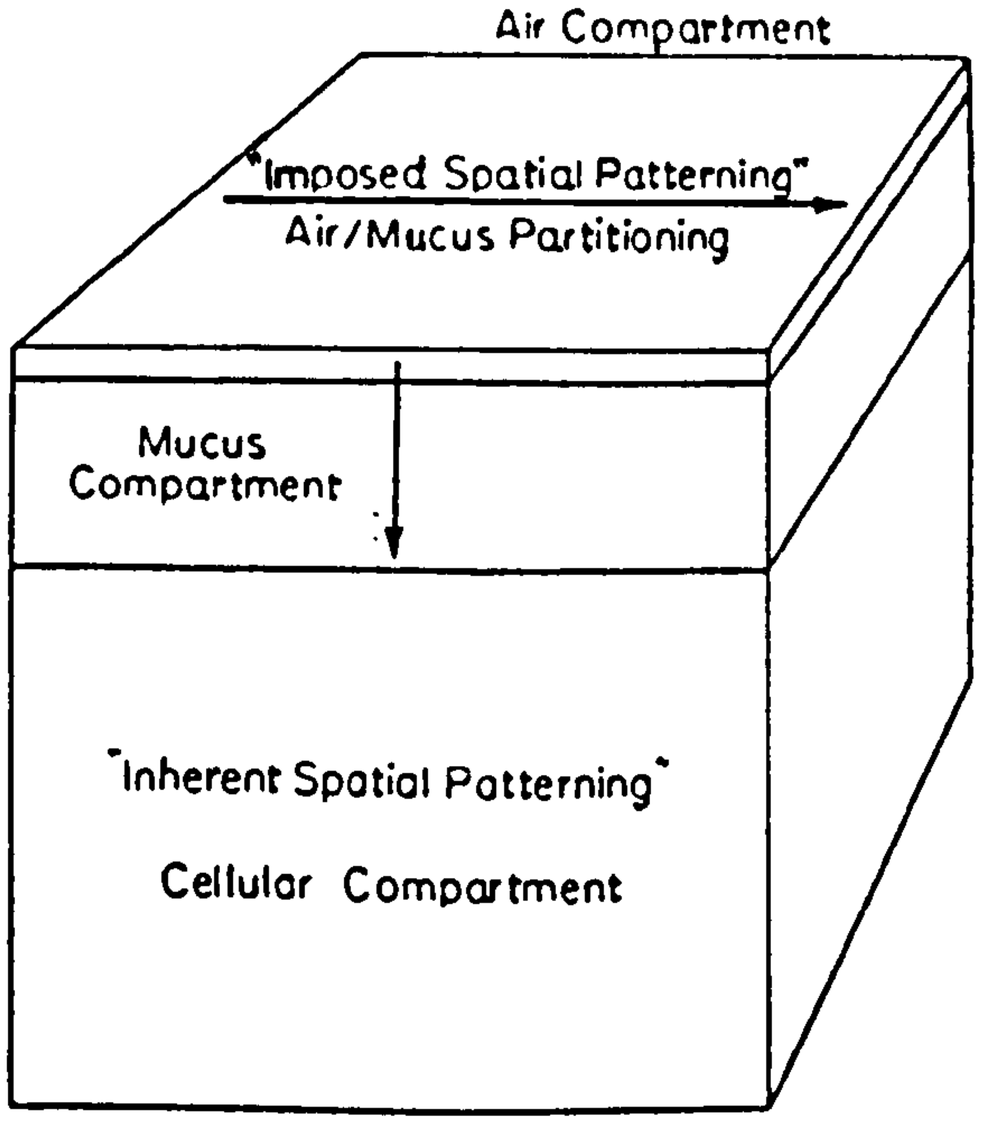


Fig. 1.3 "Imposed" and "Inherent spatial patterning". Imposed spatial patterning occurs as the odorant moves from the air compartment above the olfactory surface to the mucus compartment. Inherent spatial patterning is a characteristic of the cellular compartment containing the sensory neuroepithelium.

Examination of the surface of the olfactory mucosa *in-vivo* shows that the mucus is being transported towards the internal nares. Estimates of rates of this mucociliary transport have appeared in the literature; they range from 10 mm/min in higher animals[8] to 60 mm/min in the frog[21]. Considerations of mucociliary transport indicate that the

rates may be important factors in the access of odorants by diffusion to receptor sites on the receptor cell membranes.

The interaction of an odorant with a receptor molecule is considered to be a reversible reaction. However, the mechanism of odorant removal is not yet clear. When the odorant is removed from the air over the epithelium after a long stimulation, the sensory response diminishes from tonic level to zero within a few hundred milliseconds[31]. Such relatively fast turnoff is essential for a sensory mechanism that responds to changes occuring between consecutive sniffs.

Several mechanisms for the removal of odorants from the mucoid environment have been postulated[10]. These processes, the first two of which are non-odorant specific, may act alone or in combination and may be summarised as: (i) mucociliary transport of odorants towards the internal naris, (ii) fluid-phase pinocytotic uptake of odorants by sustentacular cells and receptor neurons, (iii) a mechanism mediated by receptor molecules in the membrane of the sustentacular cells and receptor neurons, resulting in internalisation and subsequent catabolism of the odorant, (iv) internalisation and catabolism of the receptor as well as the odorant.

Dahl et al.[32] found that some odorants were modified by the cytochrome P-450 oxidative enzyme system thereby sugesting a possible mechanism for odorant removal. Another likely mechanism may be odorant dilution from the mucus layer into

the much larger volume of the underlying tissue, followed by clearance in the blood[33].

1.3 ELECTROPHYSIOLOGY

Several techniques have been employed to monitor excitation of olfactory receptor cells by odorants (see Fig. 1.2, p4). Ottoson in 1956 introduced the electro-olfactogram (EOG) which measures summated potentials from the surface of the epithelium[34]. The EOG responses involve transepithelial currents carried through the apical membrane by sodium and possibly potassium ions, and require extracellular calcium[35]. It is widely accepted that the EOG represents chiefly a summation of generator potentials in the olfactory neurons, similar to those observed in other sensory cells[24]. It is easily obtainable and thus constitutes a useful experimental tool in physiological, physicochemical, and biochemical analyses of olfactory mechanisms. However, the EOG is also influenced by secretory activity and does not provide information on individual olfactory sensory neurons.

Several investigators have used extracellular recording (mainly with metal-filled micropipettes) to measure the frequency of spikes elicited by odorants in single sensory neurons[36]. These studies show that individual sensory neurons are responsive to many, but not all, odorants and that each neuron responds to a different spectrum of odorants. Thus, different odorants activate distinct populations of olfactory

sensory neurons sending unique patterns of neuronal activity to the central nervous system.

Intracellular recordings from olfactory sensory neurons are difficult due to the small size of the neuron stoma, but have been carried out[37]. These recordings demonstrate that the sensory neurons have a relatively high input resistance (ca. 200 MOhms) and low resting potential (-45 +/- 15 mV); that the neurons depolarise, decrease their input resistance and increase their firing rate in response to most odorants; that these changes increase with odorant concentration; and that membrane conductances to sodium and possibly to potassium are involved.

The neurotransmitter(s) at the first synapse in the olfactory pathway has not been unequivocally identified. The dipeptide carnosine (β-alanyl histidine) fulfills many of the required criteria[38], but there is still a controversy with respect to its ability to affect the postsynaptic neurons[39]. Several other types of neurotransmitter receptors (e.g. α- and β-adrenergic, muscarinic cholinergic and peripheral type benzodiazepine receptors) have been found in olfactory epithelium tissue[38,40]. Some may be related to pre-receptor events such as mucosecretory activity[10]. Acetylcholine and substance-P have been reported to be potent, odorant-like stimulatory ligands[41].

1.4 BIOCHEMISTRY OF OLFACTORY RECEPTION

1.4.1 Site of Transduction

In olfaction, the process of recognition, transduction, and encoding of odorants occur in a single cell, the olfactory sensory cell. There are several lines of evidence to suggest that recognition sites for odorants are on proteins. Firstly, the existence of specific anosmias[42] implies that there are highly specific receptor sites with which all members of an odour class react when they are used as stimuli at low concentrations. It seems unlikely that such specificity could be achieved without proteins being implicated as receptor sites. An electrophysiological study[43] of cross-adaptation to various odorants by olfactory sensory neurons, suggests that there are receptor sites that are specific for each of the odorants used, in addition to receptor sites of low specificity. Another line of evidence that olfactory receptor sites are proteinaceous is the high degree of correlation of molecular size and shape with odour quality[44]. Compounds with similar odour quality tend to have similar molecular dimensions implying that there are receptor sites into which they fit, analogous to the fit of substrates into sites on enzymes.

However, despite much effort, the proteins that bind odorants and generate the sensory signal have not been identified as distinct molecular species[5,6]. This, together

with the possibility that odorants (mostly lipophylic) could generate membrane currents by directly interacting with the lipid bilayer, led some workers to suggest that olfactory receptor proteins may not exist[45].

The search for odorant receptors in the past has primarily employed binding studies and affinity labelling experiments. Unfortunately, the hydrophobic nature of most odorants and the difficulty of correlating binding with function in the same system have thus far prevented the unambiguous identification of bona fide odorant receptor proteins. Using radioactive odorants, proteins that bind camphor[46], anisole[47], and pyrazines[48] have been identified in homogenates of olfactory tissue, but none of these has been shown unambiguously to play a role in olfactory reception. The best characterised of these odorant binding proteins is a pyrazine binding protein from bovine nasal tissue. However, this protein appears to be a component of the mucus and to originate from glands in both the olfactory and respiratory epithelium[49]. This, and the fact that many structurally unrelated odorants bind with similar low affinity to this protein, suggest that it is unlikely that this mucus component plays a role in olfaction. It is thought to act probably as an odorant carrier or scavenger facilitating access to or removal from the receptors.

It is believed that the cilia on the olfactory neurons are the morphological elements most likely to be responsible

for transducing olfactory stimulation into nervous activity. Ciliation increases the membrane area of the neurons many-fold, and the cilia are the first features in the epithelium that an odorant molecule would encounter, since their position in the mucus places them closest to the external environment. It has been shown[50] that cilia bear membrane particles at high density which are putative receptor sites. Ottoson[51] has used Shibuya's[52] experiment of applying absorbent paper to the epithelium to abolish the EOG, as evidence that the cilia are the sensory receptors, since he argues that such treatment must remove cilia. Bronshtein and Minor[53] showed that brief exposure of the olfactory epithelium of the frog to 0.1-0.15 % solutions of Triton x-100 resulted in destruction of cilia and decline of the EOG response to chemical stimuli. Electron microscopy revealed that the cilia had been removed. After 2-3 days the olfactory cilia had regenerated; during this regeneration period the EOG response was gradually restored.

More recently, isolated cilia preparations have been shown to have amino acid odorant binding activity[4,17] and high concentrations of transductory enzymes[54]. A preparation of olfactory cilia from the frog, Rana ridibunda[55], was shown to contain several specific proteins, four of which were glycosylated. Similar results were reported in another frog species, Rana catesbeiana[56]. In view of the effect of lectins on olfactory responses, it was proposed that one or more of them was a functional surface component. One ciliary glycoprotein, gp95 (molecular weight 95 kD) found abundantly

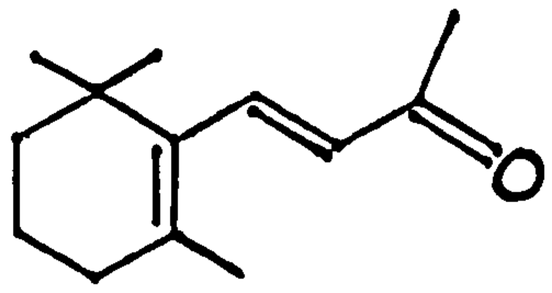
in the cilia, is believed to be a plausible candidate for an olfactory receptor protein[57]. Thus far, however, no interaction with odorants or other functional activity related to olfaction has been reported for this protein.

The supporting cells cannot be the receptors in the olfactory organ, since when they are isolated in the degenerating epithelium they do not respond to odorants. The terminal swellings of the neurons are not excluded from a role in transduction and may perform the function of detecting odours with the cilia. However, it has not yet proved possible to isolate components of the olfactory epithelium and thus unequivocally allot functions to them. Therefore, the position of the cilia as the site of olfactory transduction remains a matter for debate.

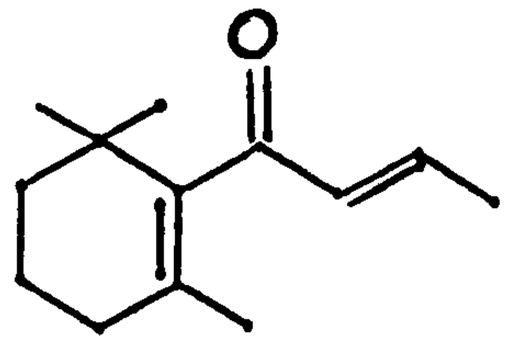
1.4.2 Olfactory Receptor Diversity

The olfactory system of terrestrial vertebrates is capable of responding to practically all volatile organic compounds. Different cells are activated by different odorants, but all cells respond in the same way, i.e. depolarisation and action potentials. In other receptor systems, only a few members of any given group of structurally related ligands can serve as agonists. In contrast, practically any chemical modification of a given odorant will yield another odorant, though often with a different perceived quality. For instance, β-Ionone (1) has the characteristic

fragrance of violets, whereas β-Damascone (2) in equal concentration exhibits a completely different and complicated odour profile in which fruity-flowery, exotic-spicy and chrysanthemum-like elements predominate[58].

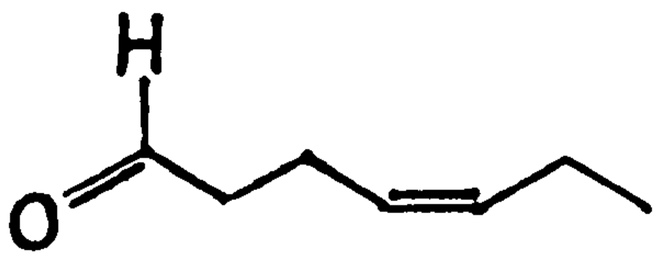


(1)

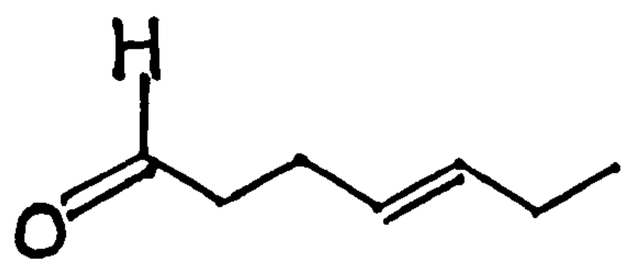


(2)

Similarly, at very low concentrations (1 ppb) (Z)-4-heptanal (3) has a creamy butter flavour compared to an agressive putty and green odour of the unsaturated heptanal E-isomer (4)[58].

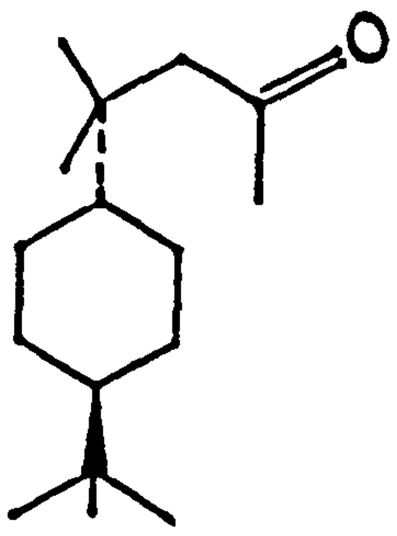


(3)

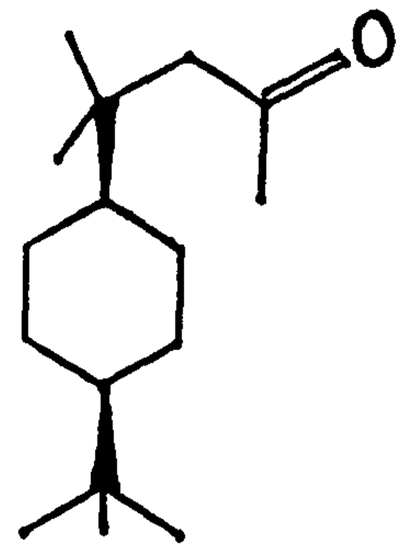


(4)

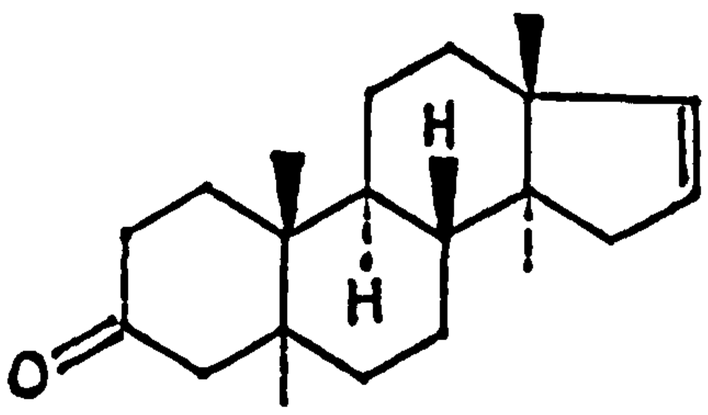
Conversely, structurally unrelated types of compounds may show similar odour characteristics. A remarkable example is that of the diastereoisomeric ketones (5) and (6). The cis-compound (6) possesses the same penetrating urine odour as the steroids (7) and (8), while its trans-epimer (5) is odourless[58].



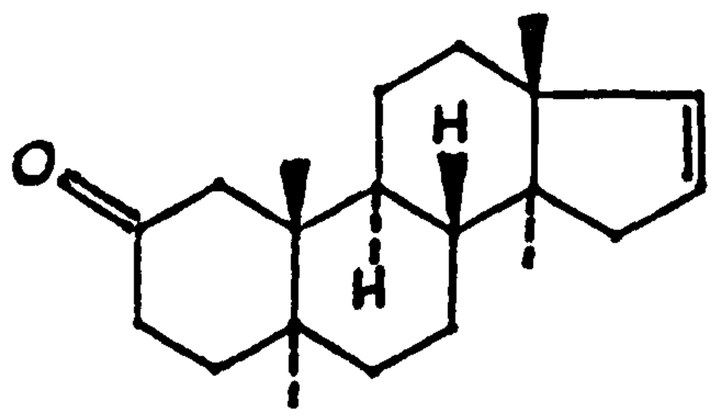
(5)



(6)



(7)



(8)

The ability of the olfactory receptor to recognize such a diversity of odorants may be simply explained by the existence of a family of receptor molecules, having different odorant binding sites in a "variable region" of the molecule but sharing the ability to interact with common transduction components of a molecular "constant region"[47,55]. Fig.1.4 illustrates such a view of olfactory reception. Such a design is analogous to that of the immune system[55].

The size of the olfactory receptor "repertoire" is not known. Data on human specific anosmias indicate the existence

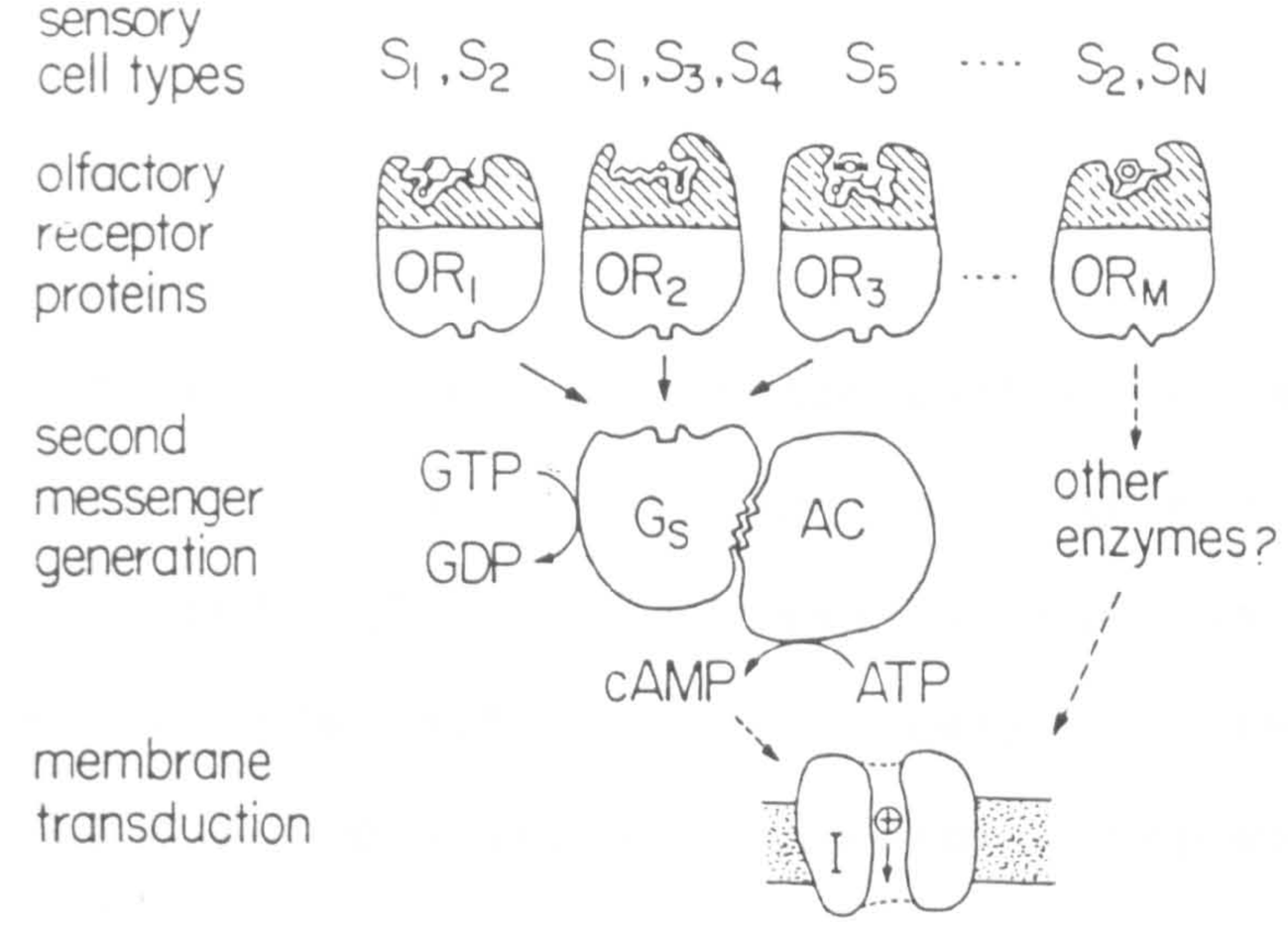


Fig. 1.4

A schematic view of olfactory reception. At least a few dozen (M) olfactory receptor molecule (OR) types may exist, present on the dendrites of a similar number (N) of sensory neuron types.

A sensory neuron (e.g. S_1) may have more than one type of receptor molecule (in this case OR_1 and OR_2). A given OR molecule (e.g. OR_2) may be present on more than one sensory neuronal type (in this case S_1, S_3 and S_4). Alternatively, clonal exclusion may pertain, where a one-to-one relationship will hold (e.g. sensory neuron S_5 and OR molecule OR_3). Many OR molecule types may converge (in different cells) on a common transduction machinery, the best candidate being G_s and adenylate cyclase (see text). cAMP (or other messengers produced by parallel transduction enzymes) would then activate the sensory ion channels to produce neuronal membrane depolarization.

[From Lancet, D. and Pace, U. (1987) TIBS, 12, p63.]

of at least a few dozen receptor types[60]. Based on ligand affinity considerations it has been argued[61] that the upper limit for the olfactory repertoire may be 10^2-10^4, cosiderably smaller than the immunoglobulin repertoire 10^7-10^9 [62]. Intriguingly, colour vision operates with only 3 photo-receptor types[63], possibly because absorption spectra of organic chromophores can easily cover one-third of the entire visible wavelength range, whereas a typical protein receptor would usually bind only a much smaller fraction of all possible ligands.

The neuronal membrane receptors that transduce odorant signals have not yet been identified. The difficulties in achieving this goal may be partly attributed to the possible diversity of olfactory receptor binding sites.

1.4.3 Molecular Mechanisms

The olfactory system, in common with other major senses, processes primary stimuli through a complete interactive system of synapses. Tranduction and coding, in analogy with other receptor systems[64], are likely to be intrinsic properties of a micro-molecular receptor complex. Odorants are low-molecular-weight ligands, and are therefore most likely to exert their effect via an allosteric conformational transition[8] rather than through receptor cross linking[65]. One possible conformational mechanism is that of an odorant-gated ion channel[66]. This configuration involves little amplification (one bound ligand opens one ion channel), and may not be optimal for a sensory mechanism that requires high sensitivity. More plausibly, odorant binding would cause the activation of an enzymatic cascade, leading to changes in second messenger levels[8]. Such a transduction mechanism involves high amplification factors; morethan 10^5 second messenger molecules may be affected per one activated receptor molecule[67].

It is convenient to discuss the ideas in the context of the allosteric-membrane-enzyme (AME) hypothesis[68]. This model

takes into account the biochemical features of the olfactory system that has been implicated in alternative mechanisms and conveniently considers all the postulated mechanisms. These general biochemical features, which may be found in olfactory cilia, are shown diagramatically in Fig. 1.5.

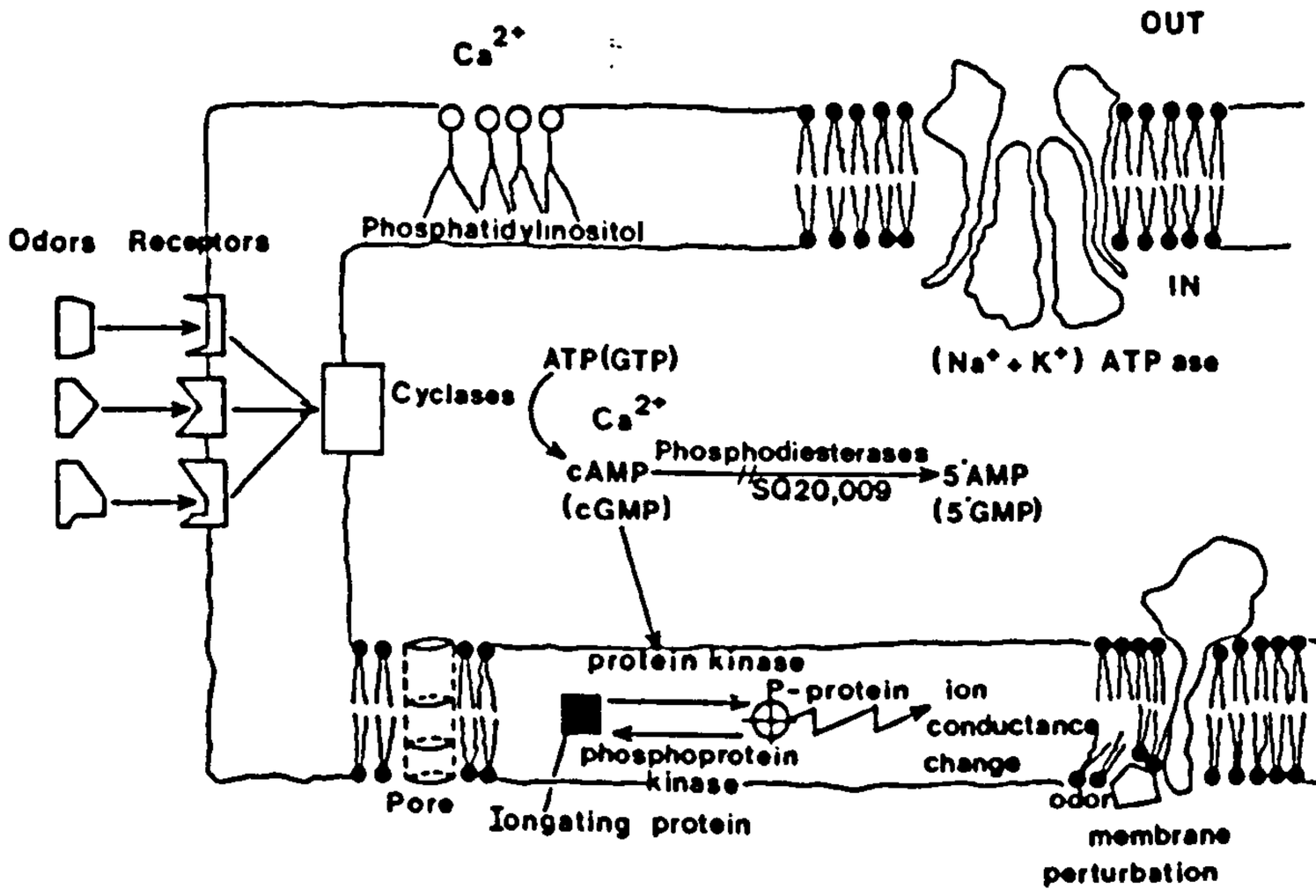


Fig. 1.5 Some general biochemical mechanisms suggested in olfactory cilia. [From Dodd, G. & Persaud, K.[85]]

Some of the features of this AME hypothesis are: (i) The binding sites for odorants may be proteins or phospholipids and may exhibit different degrees of selectivity to different odorants, with possibly high binding specificity to some odorants of biological significance, such as pheromones. (ii) The receptor potential is brought about by ligand-initiated

conformational change in an ion-gating protein, by any of the following mechanisms: (a) modulation of membrane fluidity, with consequent alteration in membrane phospholipid-protein interactions; (b) direct ligand-induced conformational change of an allosteric protomer of a membrane enzyme with intrinsic ion-gating properties such as a Na^+ ,K^+-ATPase or a Ca^{2+}-ATPase; (c) modulation of a second messenger system through an enzyme cascade system or through noncovalent binding or covalent chemical modification of the protein. (iii) The fraction of sites occupied will mainly determine the magnitude of the receptor potential. (iv) The degree of response of different primary neurons to identical stimuli may be determined by either membrane phospholipid composition or by relative distribution of receptor proteins.

An important feature of the model is that some degree of signal processing is expected directly at the membrane level. Natural olfactory stimuli generally consist of complex mixtures of odorants, and these can be expected to bind to several types of receptors in the membrane. Interaction between these receptors through lipid-lipid interactions. lipid-protein interactions, or protein-protein interactions in an oligomeric complex can be expected to contribute to the establishment of a primary information pattern for a particular odour stimulus.

The AME hypothesis considers that ciliary membranes are unlikely to have properties different from the other well-

described biological membranes and membrane enzyme systems.

Odorants probably act as regulatory ligands of a membrane-enzyme system which is coupled to the ionophoric proteins in the plasma membrane of the neurons. Adenylate and guanylate cyclases[69] and Na^+, K^+, Mg^{2+}-ATPase[70] are candidate enzyme systems.

1.4.4 CURRENT MODELS FOR OLFACTORY TRANSDUCTION

1.4.4.i Second Messenger

Considerable evidence has accumulated over the last decade suggesting that cyclic-AMP is involved in olfactory transduction: Olfactory epithelium has a relatively high level of adenylate cyclase activity[71]; its phosphodiesterase isozyme pattern changes when the neurons degenerate[72]; cAMP and its dibutyryl derivative (but not cGMP or its analogues) cause the appearance of electro olfactogram-like potentials; and phosphodiesterase inhibitors modulate the electro physiological response to odorants[73,74].

More recently, the last of the Sutherland's criteria for cyclic nucleotide mediation[75] was fulfilled by the demonstration that a cell-free olfactory preparation has an extremely high activity of adenylate cyclase (greater than that found in brain membranes), which is specifically enhanced

by odorants[54,76]. These findings establish the role of cyclic AMP as a second messenger in olfactory transduction. Odorant-induced transductory events may result also in the modulation of other membrane enzymes, such as the Na/K ATPase[77].

Odorant stimulation of olfactory adenylate cyclase occurs in the presence of GTP[53,76] thus suggesting the involvement of a signal-coupling GTP-binding protein or G-protein[67,78]. The olfactory G-protein can be directly identified in olfactory (but not in respiratory) cilia, via labelling with cholera toxin[54].

1.4.4.ii cAMP and Ion Channels

The major intracellular target of the second messenger cAMP is cAMP-dependent protein kinase. This enzyme has recently been identified in olfactory cilia and in deciliated epithelium membranes[78].

Electrophysiological and biochemical data support the notion that changes in intracellular cAMP modulate membrane ion conductance. Yet little is known about the nature of the ion channels involved or about the mechanism of their modulation by cAMP. Odour stimulation leads to depolarisation and increased membrane conductance in olfactory neurons[80] suggesting the opening of cation channels. The best candidate for the transduction current is sodium, but potassium has also been implicated.

In addition to acting through phosphorylation by cAMP-dependent protein kinase, cAMP could act through direct allosteric modulation of ion channels, by analogy with the function of the cGMP-gated sodium channels of retinal rod outer segment[88] (see Fig. 1.6). Evidence for the latter mechanism has recently been obtained through single ion channel recordings[81]. Besides stimulating the olfactory adenylate cyclase, odorants have also been shown to activate ion channels[88].

The discovery of an odorant-sensitive GTP-dependent adenylate cyclase and the presence of odorant-gated ion channels in olfactory cilia have led to several working hypotheses for the molecular pathways that may mediate signal transduction in olfaction. These are illustrated schematically in Fig. 1.7. One model suggests that cAMP generated by the olfactory adenylate cyclase causes the opening of an ion channel either directly or by phosphorylation of the channel via a cAMP-dependent protein kinase[81]. In this scheme generation of cAMP leads to excitation of the olfactory receptor cell. Alternatively, odorants may cause activation of the olfactory receptor cell by directly opening ion channels. In this model, phosphorylation of the channel via a cAMP-dependent protein kinase would cause its inactivation and, thus lead to desensitisation of the receptor cell.

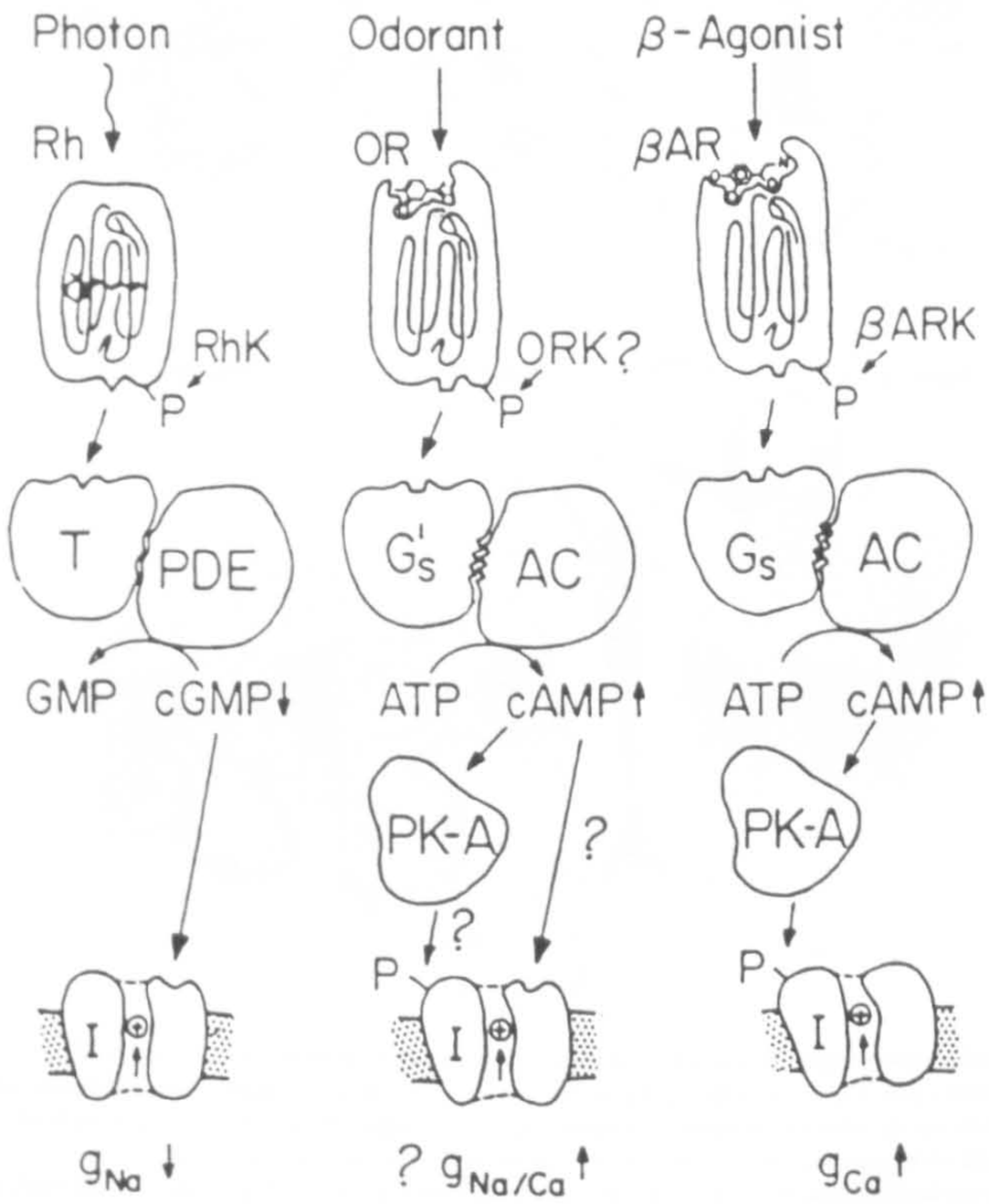


Fig. 1.6

A hypothetical view of the possible homology between olfactory receptor (OR) molecules and other cyclic nucleotide-coupled receptors, β-adrenergic receptor (βAR) and the photoreceptor protein rhodopsin (Rh). Odorant molecules serve an analogous role to β-agonists or to photons, activating a yet unidentified OR protein. OR proteins may belong to the group of membrane receptors with seven transmembrane domains that includes βAR and rhodopsin . Like βAR, OR proteins activate a G_s–adenylate cyclase system, increasing the production of cAMP from ATP. Analogously, photolysed rhodopsin activates a transducin–phosphodiesterase system to increase the breakdown of cGMP. Two distinct mechanisms mediate the modulation of ion channel conductance by cyclic nucleotides: In some neurons, cAMP may cause the opening of ion channels by activating cAMP-dependent protein kinase which catalyses protein phosphorylation. . In rod outer segments cGMP activates a cation conductance by directly interacting with an ion channel . One or both of these mechanisms may underlie the odorant-related activation of a cation conductance in olfactory neurons. OR proteins could undergo phosphorylation by an olfactory receptor-specific kinase (labelled ORK), hypothesized to be homologous to βAR kinase (βARK) and to rhodopsin kinase (RhK).

[From Lancet, D. and Pace, U. (1987) TIBS 12, p65]

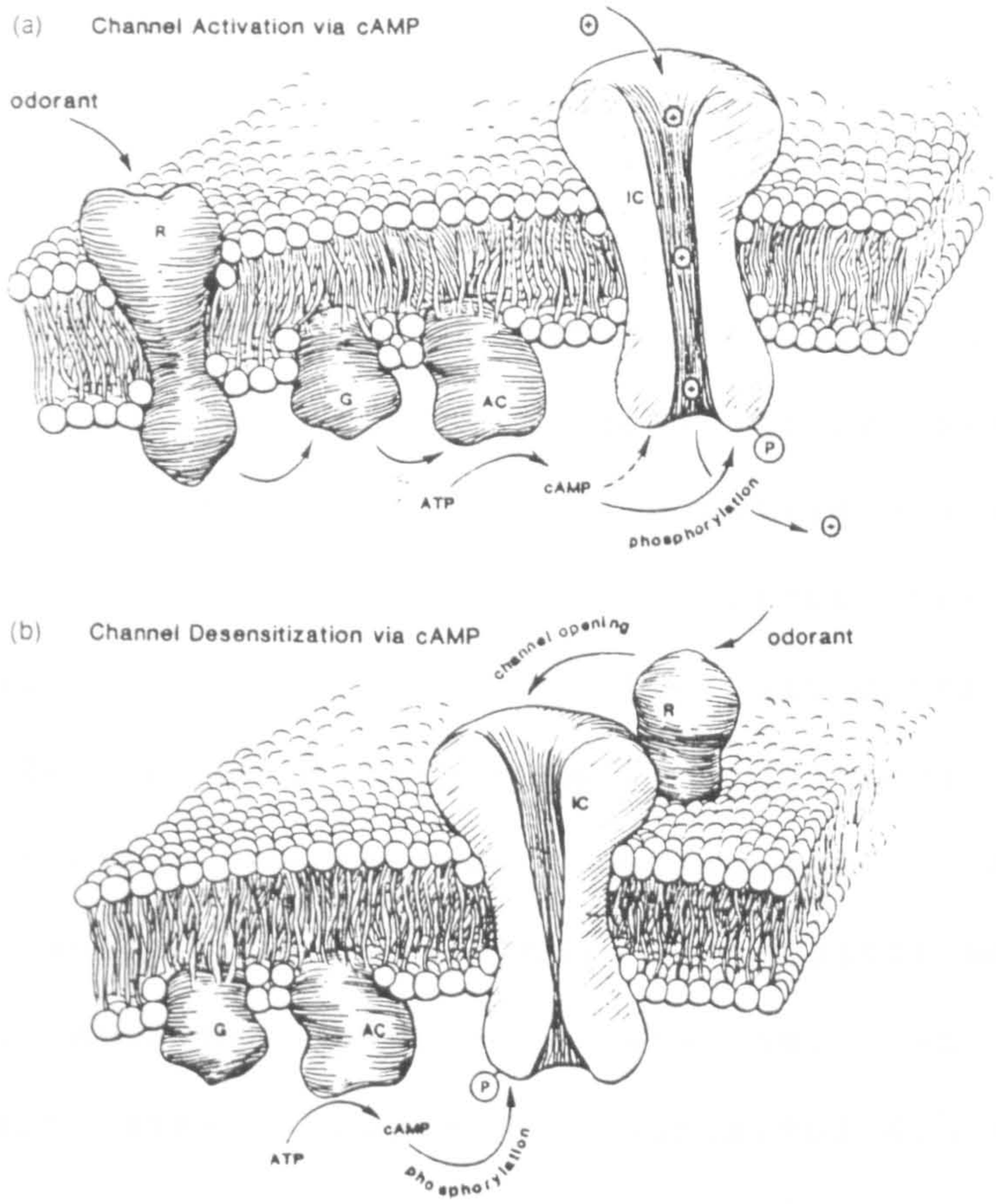


Fig. 1.7

Schematic representation of two alternative working hypotheses for signal transduction at the olfactory membrane. (a) Linkage of an odorant receptor (R) via a G protein (G) to adenylate cyclase (AC) results in the generation of cAMP which, either directly or via phosphorylation, activates an ion channel (IC).

(b) Linkage of an odorant recognition site to a channel causes opening of the channel directly. In this model, cAMP-dependent phosphorylation would lead to inactivation (desensitization) of the channel. Activation by the odorant of the olfactory adenylate cyclase may either be mediated via a distinct receptor protein or via direct activation (i.e. partial dissociation) of the G protein , as a result of partitioning of the odorant in the membrane.

[From Anholt, R.R.H. (1987) TIBS 12, p61]

It is of interest to note that odorants activate channels at nanomolar concentrations and that the generation of cAMP is observed over a longer time scale at approximately 1000-fold higher odorant concentrations. This and the observation that phosphodiesterase inhibitors reduce rather than increase the amplitude of the EOG[82] argues in favour of

the second model. However, too little information is at present available to determine which of these two models is more correct.

1.4.4.iii Other Possible Transduction Mechanisms

Many odorants (notablythose in the fruity, floral, minty and herbaceaous classes) activate adenylate cyclase, but other physiologically active odorants (e.g. putrid aliphatic acids, amines and organic solvents) do not affect the enzyme in isolated cilia[82]. Thus, it is possible that certain olfactory receptor molecules may generate an intracellular signal through different transduction mechanisms. A possible mechanism is modulation of phosphatidylinositol metabolism[83], supported by evidence whereby L-alanine, an amino acid odorant, activates phosphatidylinositol-4,5-bisphosphate phosphodiesterase (phospholipase C) in isolated fish olfactory cilia[84]. Phospholipase C liberates two second messengers, inositol tris-phosphate and diacylglycerol, the latter activating protein kinase C. Such kinase activity was investigated in olfactory membranes, with conflicting results. Anholt et al.[85] identified protein kinase C in frog olfactory cilia by immunoblot analysis and by phorbol-ester binding. However, neither they, nor Heldman and Lancet[78], could demonstrate protein kinase C activity by phosphorylation assays in the presence of calcium and phosphatidyl serine.

REFERENCES

1. Shepherd, G.M. (1976) "Handbook of Physiology", ed. Kandel. E.R. (Am. Physiol. Soc., Bethesda, MD), Sect. 1, Vol. 1, pp. 945-968.

2. Gesteland, R.C. (1971) "Handbook of Sensory Physiology", ed. Beidler, L.M. (Springer, Berlin), Vol. 4, Pt. 1, pp. 132-150.

3. Getchell, T.V. (1974) J. Gen. Physiol., 64, 241-261.

4. Rhein, L.D. & Cagan, R.H. (1980) Proc. Natl. Acad. Sci. USA, 77, 4412-4416.

5. Price, S. (1981) "Biochemistry of Taste and Olfaction, eds. Cagan, R.H. & Kare, M.R. (Academic, New York), pp. 69-84.

6. Dodd, G. & Persaud, K. (1981) "Biochemistry of Taste and Olfaction, eds. Cagan, R.H. & Kare, M.R. (Academic, New York), pp. 333-358.

7. O'Brian, D.F. (1982) Science, 218, 961-966.

8. Moulton, D.G., Beidler, L.M. (1967) Physiol. Rev., 47, 1-52.

9. Graziadei, P.P.C. (1971) Handb. Sensory Phsiol., ed. Beidler, L.M., Sect. 1, Vol. 4, pp. 27-58.

10. Getchell, T.V., Margolis, F.L., Getchell, M.L. (1984) Progr. Neurobiol., 23, 317-345.

11. Margolis, F.L. (1985) Trends Neurosci., 8, 542-546.

12. Burd, G.D. Davis, B.J. Macrides, F., Grillo, M., and Margolis, F.L. (1982) J. Neurosci., 2, 244-255.

13. Lindquist, N.G., Lyden, A., Narfstrom, K., and Samaan, H. (1983) Experienta, 39, 797-799.

14. Schwob, J.E., Farber, N.B. and Gottlieb, D.I. (1986) J. Neurosci., 6, 208-217.

15. Graziadei, P.P.C. & Monti Graziadei, G.A. (1979) J. Neurocytol., 8, 1-18.

16. Simmons, P.A., Getchell, T.V. (1981) J. Neurophysiol., 45, 516-528.

17. Rhein, L.D., Cagan, R.H. (1981) "Biochemistry of Taste and Olfaction, eds. Cagan, R.H. & Kare, M.R. (Academic, New York), pp. 47-68.

18. Graziadei, P.P.C. (1966) J. Zool. Lond., 149, 89-94.

19. Kratzing, J.E. (1970) Anst. J. Biol. Sci., 23, 447-458.

20. Okano, M., Weber, A.F., and Frommes, S.P. (1967) J. Ultrastruct. Res., 17, 487-502.

21. Reese, T.S. (1965) J. Cell Biol., 25, 209-230.

22. Lidow, M.S., Menco, B.Ph.M. (1984) J. Ultrastruc. Res., 86, 18-30.

23. Menco, B.P.M., Dodd, G.H., Davey, M., and Bannister, L.H. (1976) Nature, Lond., 263, 597-599.

24. Fuortes, M.G.F. (1971) Handb. Sensory Physiol., ed. Lowenstein, W.R., Vol. 1, pp. 246-268.

25. Abramson, M. & Harker, L.A. (1973) Otolaryngot Clin. N. Am., 6, 623-635.

26. Bostock, H. (1974) "Transduction Mechanisms in Chemoreception", ed. Poynder, T.M. (London, I.R.L.), pp. 27-38.

27. Mozell, M.M., Sheehe, P.R., Swieck, S.W. Jr., Kurty, D.B., and Horning, D.E. (1984) J. Gen. Physiol., 83, 233-267.

28. Moulton, D.G. (1976) Physiol. Rev., 56, 578-593.

29. Getchell, T.V., Heck, G.L., De Simone, J.A., and Price, S. (1980) Biophys. J., 29, 397-412.

30. King, M. and Viires, N. (1979) J. Appl. Physiol., 47, 26-31.

31. Getchell, T.V., Shepherd, G.M. (1978), J. Physiol., 282, 541-560.

32. Dahl, R.A., Hadley, W.M., Hahn, F.F., Benson, J.M., McClellan, R.O. (1982) Science, 216, 57-59.

33. Maruniak, J.A., Silver, W.L., and Moulton, D.G. (1983) Brain Res., 265, 312-316.

34. Ottoson, D. (1956) Acta Physiol. Scand., 35, 122, 1-83.

35. Takagi, S.F., Kitamura, H., Imai, K., and Takenchi, H. (1969) J. Gen. Physiol., 59, 115-130.

36. Sicand, G., and Holley, A. (1984) Brain Res., 292, 283-296.

37. Getchell, T.V. (1979) Brain Res., 123, 275-286.

38. Margolis, F.L. (1981) "Biochemistry of Taste and Olfaction, eds. Cagan, R.H. & Kare, M.R. (Academic, New York), pp. 369-394.

39. Frosch, M.P., Dichter, M.A. (1984) Brain Res., 290, 321-332.

40. Anholt, R.H., Murphy, K.M.M., Mack, G.E., and Snyder, S.H. (1984) J. Neurosci., 4, 593-603.

41. Bouvet, J.F., Holley, A. (1984) CR Acad. Sci. Paris, 298 (ser. 3), 169-172.

42. Amoore, J.E. (1977) Chem. Senses Flavor, 2, 267-281.

43. Baylin, F. and Moulton, D.G. (1979) J. Gen. Physiol., 74, 37-55.

44. Ammore, J.F., Palmieri, G., and Wanke, E. (1967) Nature, Lond., 216, 1084-1087.

45. Kashiwayanagi, M., Kurihara, K. (1984) Brain Res., 293. 251-258.

46. Fesenko, E.E., Novoselov, V.I., and Krapivins-Kaya, L.D. (1979) Biochim. Biophys. Acta, 587, 424-433.

47. Goldberg, S.J., Turpin, J., and Price, S. (1979) Chem. Senses Flavor, 4, 207-214.

48. Pelosi, P., Baldaccini, N.E., and Pisanelli, A.M. (1982) Biochem. J., 201, 245-248.

49. Pevsner, J., Sklar, P.B., and Snyder, S.H. (1986) Proc. Natl. Acad. Sci., USA, 83, 4942-4946.

50. Menco, B.P.M. (1978) Abstracts 3rd ECRO Congress, Pavia.

51. Ottoson, D. (1971) Handb. Sens. Physiol., ed. Beidler, L.M., Part 1, Vol. 4, pp.95-131.

52. Shibuya, T. (1964) Science, Wash., 143, 1338-1340.

53. Bronshtein, A.A. and Minor, A.V. (1977) Tsitologiya, 19, 33-39.

54. Pace, U., Hanski, E., Saloman, Y., and Lancet, D. (1985), Nature, 316, 255-258.

55. Chen. Z. and Lancet, D. (1984) Proc. Natl. Acad. Sci., USA, 81, 1859-1863.

56. Anholt, R.H., Aebi, U., and Snyder, S.H. (1986) J. Neurosci., Vol. 6, 1962-1969.

57. Chen, Z., Pace, U., Ronen, D., and Lancet, D. (1986) J. Biol. Chem., 261, 1299-1305.

58. Ohloff, G. (1986) Experientia, 42, 271-279.

59. Sigal, N.H., Klinman, N.R. (1978) Adv. Immunol., 26, 255-337.

60. Amoore, J.E. (1971) Handb. Sens. Physiol., ed. Beidler, L.M., Part 1, Vol. 4, pp. 245-256.

61. Lancet, D. (1986) Annu. Rev. Neurosci., 9, 329-355.

62. Hunkapiller, T. and Hood, L. (1986) Nature, 323, 15-16.

63. Nathan, J., Thomas, D., and Hogness, D.S. (1986) Science, 232, 193-202.

64. Dodd, G.H. (1974) "Transduction Mechanisms in Chemoreception", ed. Poynder, T.M. (London, I.R.L.), pp.103-113.

65. Cuatrecasas, P., and Roth, T. eds. (1983) Receptor Mediated Endocytosis. Receptors and Recognition series, Vol. B15, London-New York, Chapman & Hall, pp. 304.

66. Vodyanoy, V. and Murphy, R.B. (1983) Science, 220, 717-719.

67. Stryer, L. (1986) Annu. Rev. Neurosci., 9, 87-119.

68. Dodd, G.H., Menevse, A., and Poynder, T.M. (1977) Olfaction Taste VI, ed. Le Magnen, J. & Macleod, P. (London, I.R.L.), pp. 59.

69. Squirrel, D. (1978) Ph.D. Thesis, Univ. of Warwick, Coventry, England.

70. Dodd, G.H. (1970) Comp. Biochem. Physiol., 36, 633-637.

71. Kurihara, K., and Koyama, N. (1972) Biochem. Biophys. Res. Commun., 48, 30-34.

72. Margolis, E.L. (1975) Soc. Neurosci. Symp., 3, 167-188.

73. Menevse, A., Dodd, G., and Poynder, T.M. (1977) Biochem. Biophys. Res. Commun., 77, 671-677.

74. Minor, A.V., Sakina, N.L. (1973) Neurofysiologiya, 5, 415-422.

75. Robinson, G.A., Butcher, R.W., and Sutherland, E.W. (1971). "Cyclic AMP". New York-London, Academic, pp.531.

76. Shirley, S.G., Robinson, C.J., Dickenson, K., Aujla, R., and Dodd, G.H. (1986) Biochem. J., 240, 605-607.

77. Dreeson, T.D., and Koch, R.B. (1982) Biochem. J., 203, 69-75.

78. Gilman, A.G. (1984) Cell, 36, 577-579.

79. Heldman, J., and Lancet, D. (1986) J. Neurochem., 47, 1527-1533.

80. Getchell, T.V. (1986) Physiol. Rev., 66, 772-817.

81. Nakamura, T. and Gold, G.H. (1987) Nature, 325, 442-444.

82. Sklar, P.B., Anholt, R.R.H., and Snyder, S.H. (1986) J. Biol. Chem., 261, 15538-15543.

83. Berridge, M.J., and Irvine, R.F. (1984) Nature, 312, 315-321.

84. Huque, T., and Bruch, R.C. (1986) Biophys. Biochem. Res. Commun., 137, 36-42.

85. Anholt, R.R.H., Mumby, S.M., Stoffers, D.A., Girard, P.R., Kuo, J.F., Gilman, A.G., and Snyder, S.H. (1987) Biochemistry, 26, 788-795.

CHAPTER 2

OLFACTORY ADENYLATE CYCLASE OF THE RAT

2.1 INTRODUCTION

2.1.1 THE ADENYLATE CYCLASE SYSTEM

An enzyme known as adenylate cyclase is present in the plasma membrane of most cells. In the presence of magnesium ions this enzyme catalyses an intra-molecular condensation of ATP in the cytoplasm to produce cyclic adenosine-3,5-monophosphate (cyclic AMP). The reactions are shown in Fig. 2.1

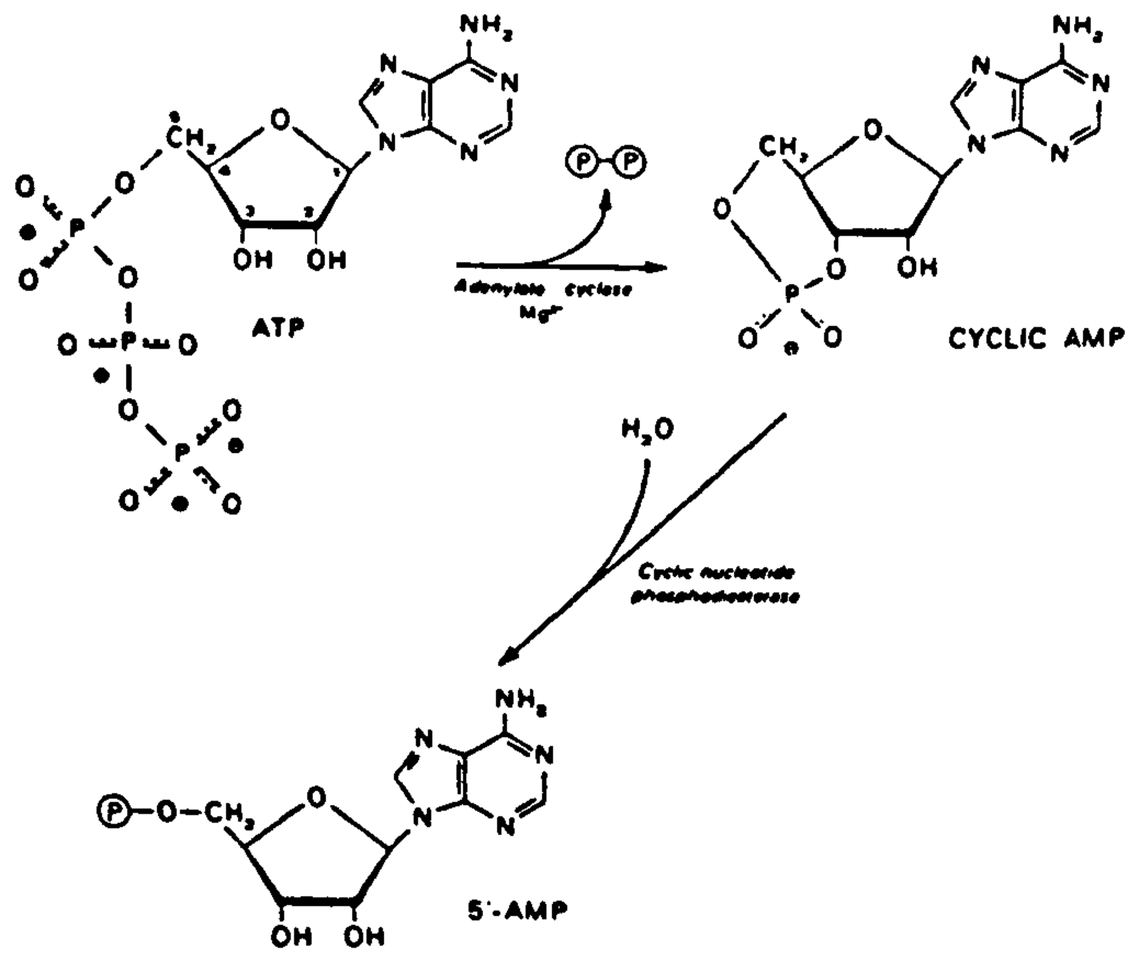


Fig. 2.1 The formation and inactivation of cyclic AMP.

The adenylate cyclase system consists of three classes

of component (see Fig. 2.2). The receptor (R) is located at the outer membrane surface and contains a specific site for binding of hormones and neuotransmitters. The catalytic unit (C) and the guanine-nucleotide binding regulatory component (G) are at the inner face of the membrane. The latter contains site(s) for binding GTP and is responsible for mediating the effects of GTP and the various hormones on the activity of C. Different types of G units (termed G proteins) have been distinguished functionally (described in section 2.1.3).

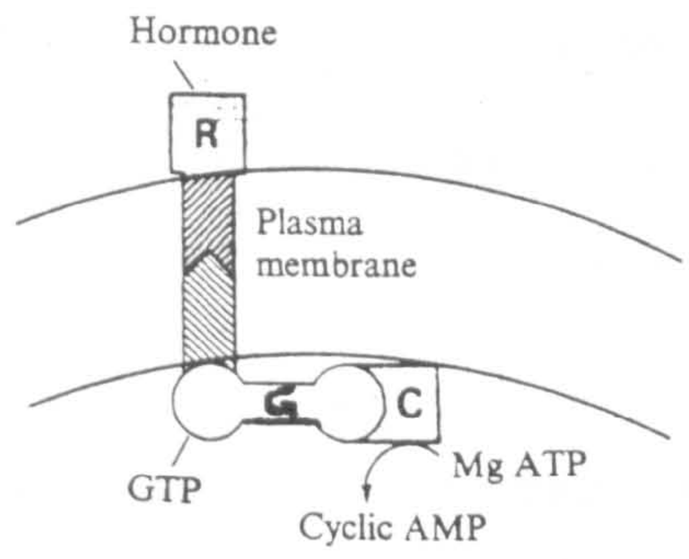


Fig. 2.2 Schematic representation of the components and organisation of the adenylate cyclase holoenzyme responsible for regulation by hormones and GTP. The receptor (R) is visualised as spanning the plasma membrane and having different segments (indicated by the shaded and cross-hatched areas) which have functions for binding of hormone, attachment to membrane and linkage with the nucleotide regulatory unit (G) that binds GTP. The G unit forms a bridge between R and the catalytic component (C) at the internal face of the membrane.

[From Rodbell, M. (1980) Nature, 284, p18]

It has now become clear that the receptors with which many hormones combine are closely related to, or may even be part of, an adenylate cyclase system. In these cases, depending upon whether the cyclase is stimulated or inhibited, the result of the hormone-receptor interaction (i.e. the

stimulus) will be an increase or a decrease in the intracellular level of cyclic AMP. When the stimulus is an increase in the level of cyclic AMP, then the hormone might be regarded as a first mesenger, with cyclic AMP as the second messenger.

The first messenger carries the required information to the cell, and the second messenger transfers this information to the cells' internal machinery. The end result of the increased level of cyclic AMP will depend entirely on the nature of the cells in which it occurs and on the prevailing conditions. Cyclic AMP is inactivated by a relatively specific family of cyclic nucleotide phospho-diesterases which catalyse its hydrolysis to the inactive 5-AMP (see Fig. 2.1).

Much of the evidence in support of the the concept of cyclic AMP as a second messenger derives from the work of Sutherland and his colleagues[1]. Adenylate cyclase has been found in the plasma membranes of most cells examined; the highest levels are found in nervous tissue, particularly the grey matter of the cerebral cortex. Evidence is now accumulating to show that a wide variety of hormones and bio-active amines can stimulate the activity of adenylate cyclase and so increase the cellular concentrations of cyclic AMP.

Table 2.1 lists some of the peripheral tissues for which there is evidence that cyclic AMP mediates the response to hormones. The cyclic AMP activates a number of protein kinases

which catalyse reactions leading to a variety of cellular functions.

Tissue	Hormone	Response
Liver	Adrenaline*, glucagon	Glycogenolysis, gluconeogenesis, ketogenesis, K^+ and Ca^{2+} release, enzyme induction
Heart	Adrenaline*, glucagon, prostaglandins	Glycogenolysis, increased contractile force
Skeletal muscle	Adrenaline*, prostaglandins	Glycogenolysis
Intestine	Adrenaline*	Glycogenolysis, inhibition of contractions and tone
Kidney	Parathyroid hormone	Phosphate excretion
	Antidiuretic hormone	Water reabsorption
Salivary glands	Adrenaline	Amylase secretion
Bone	Parathyroid hormone	Bone resorption and calcium release
Adipose tissue	Adrenaline*, glucagon, corticotrophin and thyrotrophin, luteinizing hormone, prostaglandin E_1.	Lipolysis
Sympathetic ganglia	Dopamine	Hyperpolarization and inhibition of transmission
Pancreatic islets	Glucagon	Stimulation of insulin secretion
Thyroid	Thyrotrophin	Thyroid hormone release
Adrenal cortex	Corticotrophin	Hydrocortisone secretion
Corpus luteum of ovary	Luteinizing hormone	Progesterone secretion
Pineal gland (amphibia)	Adrenaline*	Melatonin release and lightening of skin colour
Frog skin	Melanocyte-stimulating hormone	Melanin granule dispersal in melanophores (skin darkening)

* Action of adrenaline mimicked by isoprenaline.

Table 2.1 Some effects of hormones on tissues for which there is evidence that activation of the adenylate cyclase-cyclic AMP system may play an intermediary role. [From Bowman, W.C. and Rand, M.J. (1980) "Textbook of Pharmacology" 2nd Ed. (Blackwell Scientific Publications) p2.22]

Some of the effects of cyclic AMP may involve changes in the binding of calcium ions or in membrane permeability, which in turn trigger the response of the cell. Many tissues are highly specific in that their adenylate cyclase is activated by only one or a few hormones. For example, the adenylate cyclase in adrenal cortical tissue is stimulated by corticotrophin but not by a variety of other hormones that stimulate adenylate cyclase elsewhere. Conversely, many hormones are highly specific in that they stimulate only in a few tissues. The selectivity is presumably conferred by the presence, or the absence, of specific receptors which interact only with a particular hormone rather than by differences in

the nature of the adenylate cyclase. For example, liver membranes contain different and separate receptors for adrenalin and glucagon. That they are different is evidenced by the fact that the action of adrenalin or isoprenaline, but not that of glucagon is blocked by adrenoreceptor blocking drugs. Stimulation of either type of receptor, however, activates adenylate cyclase which is a common pathway in the reactions leading to the responses to both agents. Liver adenylate cyclase is not activated by corticotrophin; it is thought therefore that liver cells do not contain receptors for corticotrophin.

2.1.2 GUANYLATE CYCLASE

Another second messenger system involving the enzyme guanylate cyclase, which catalyses the production of cyclic guanosine monophosphate (cyclic GMP) from guanosine triphosphate (GTP), is present in some cells. It is often coupled to muscarinic receptors, which are stimulated by acetylcholine. Cyclic GMP has not been studied to the same extent as cyclic AMP. Cyclic GMP is known to play a central role in visual excitation in vertebrate retinal rod cells[2]. Sodium channels in the plasma membrane of the outer segment are kept open in the dark by a high level of cyclic GMP. The key components of the receptor-mediated cyclic GMP cascade in vision are the receptor rhodopsin, the G-protein transducin and the cyclic GMP-phosphodiesterase.

2.1.3 G PROTEINS

The G proteins are a family of signal coupling proteins which play key roles in many hormonal and sensory transduction processes in eukaryotes. G proteins carry signals from activated membrane receptors to effector enzymes and channels.

G proteins have a common design. They consist of three polypeptides: a guanyl-nucleotide binding α chain (39-52 kDa), a β chain (35-36 kDa) and a γ chain (8 kDa). G proteins cycle between an inactive GDP state to an active GTP state (see Fig. 2.3).

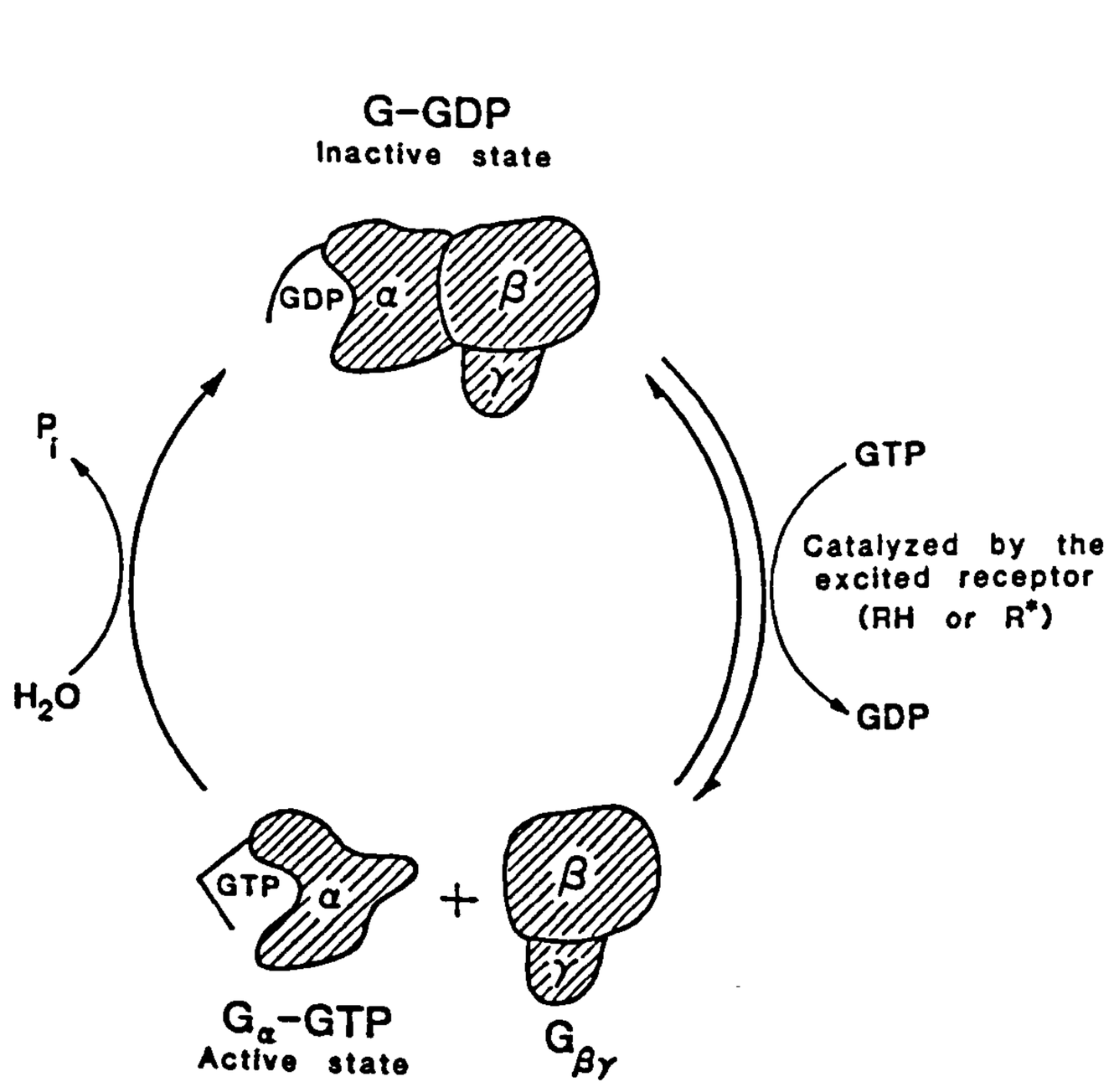


Fig. 2.3 GTP-GDP cycle of G proteins.
[From Stryer, L. (1986) Ann. Rev. Cell Biol., 2, p394]

When GDP is bound, α associates with β,γ to form a $G_{\alpha\beta\gamma}$ complex (denoted by G-GDP) that is membrane bound. When GTP is bound, the α chain (G_{α}-GTP) dissociates from the β and γ chains ($G_{\beta\gamma}$). An essential feature of G-proteins is that their conversion from the GDP to the GTP state is slow in the absence of the excited receptor (e.g a hormone-receptor complex). The role of the excited receptor is to catalyse the activation of the G protein by markedly accelerating the rate of exchange of GTP for bound GDP. G_{α}-GTP released from $G_{\beta\gamma}$ then alters the activity of the target enzyme or channel. The reaction cycle also has a built-in hydrolytic activity that converts bound GTP to GDP to terminate activation.

Evidence is mounting that G proteins are ubiquitous features of signal transduction mechanisms involving control of intracellular calcium and second messengers, regulation of cell growth, gating of ion channels, olfaction, vision and possibly other sensory systems.

A family of these G proteins has been recognised[3] and includes G_s and G_i, which respectively couple receptors to adenylate cyclase in either stimulatory or inhibitory fashion[4], transducin (TD) which couples the photon receptor rhodopsin to the activation of a cyclic GMP phosphodiesterase in vertebrate rod outer segments[5], and G_o, a guanine nucleotide binding protein of unknown function which is widely distributed and found in particularly high concentrations in brain[6]. It has recently become clear that receptors which are

coupled to the stimulation of turnover of inositol-containing phospholipids also interact with a G protein[7]; however, the identity of this transducer is not yet apparent. As with the adenylate cyclase system, certain receptors are able to inhibit turnover of inositol containing phospholipids[8] and symmetry would be maintained if a second G protein was to function to modulate this response in an inhibitory fashion. Insulin may exert some of its effect via a specific G protein distinct from those described above[8] and the p21 gene products of the ras family of oncogenes may also have a specific role in signal transduction related to diferentiation and growth control[10]. Potassium channels in cardiac pacemaker cells are thought to be opened by a G protein that is activated by the muscarinic acetylcholine receptor, which slows the cells' rate of firing[11].

The G protein family exhibits many additional structural and functional similarities. Their β chains are nearly identical, whereas their γ chains show some differences. The $\beta\gamma$ subunits of G_s, G_i, G_o and transducin are functionally interchangeable. Their respective α chains contain common regions (e.g. the site for GTP binding and hydrolysis) and distinctive regions(e.g. binding sites for receptor and effector proteins). Furthermore, the α chains of G proteins can be specifically ADP-ribosylated by bacterial toxins. G_s and transducin are targets for cholera toxin, whereas G_i, G_o, and transducin are targets for pertussis toxin.

2.1.4 OLFACTION AND THE ADENYLATE CYCLASE

The possible involvement of cyclic AMP in olfactory transduction has long been suspected. Kurihara and Koyama[12] in 1972 demonstrated the presence of high levels (ca. 570 pmoles/min/mg protein) of adenylate cyclase activity in the olfactory mucosa , but did not report any attempts to stimulate this activity with odours. Menevse et al.[13] in 1977 showed that cyclic AMP analogues and phosphodiesterase inhibitors affected the production of the EOG (electro-olfactogram, an indicator of early electrical events in odour transduction) and found that odorants did not stimulate the adenylate cyclase of olfactory membrane preparations. However, details of their preparations were not given.

In 1984 Lancet et al. of the Weizmann Institute in Israel, studied (personal communication) the properties of adenylate cyclase in isolated frog olfactory cilia, a preparation enriched in dendritic membranes of the chemosensory neurons[14]. They reported the presence of high levels of adenylate cyclase activity in this cilia preparation and demonstrated that the activity was enhanced by guanine nucleotides as well as by odorants. Their results strongly indicated the involvement of adenylate cyclase in olfactory transduction and suggested that a G protein was responsible for receptor-cyclase coupling.

In order to confirm the transductory role of a cyclase

in olfaction, the following investigations aim to study the properties of adenylate and guanylate cyclase in olfactory tissue. The rat was used as the experimental animal in an attempt to demonstrate the cyclase activity in mammals.

2.2 METHODOLOGY

2.2.1 ANIMALS

Male Wister rats of about 300g body weight were used. The animals were allowed food and water prior to the experiment.

2.2.2 MATERIALS

[8-^{3}H] cyclic AMP, [8-^{3}H] cyclic GMP, [α-^{32}P] ATP and [α-^{32}P]GTP were supplied by Amersham International; all other biochemicals were obtained from Sigma. Chemicals, except imidazole, were of analytical quality. Odorants, obtained from Aldrich Chemical Company Ltd., were of the highest commercially available quality and were used without further purification. Dowex 50W-X8-400 and chromatographic grade neutral alumina of type WN-3 were supplied by Sigma. Econo-Columns for the cyclase activity assay were obtained from Bio-Rad.

2.2.3 PREPARATION OF CRUDE OLFACTORY ADENYLATE CYCLASE

The animals were sacrificed by cervical dislocation and decapitation. The ethmoturbinates, usually 50-100 mg in weight, were removed after saggital section of the head and soaked in cold medium A (0.9% NaCl, 1mM EGTA, 5 mM phosphate buffer, pH 7.0), to remove superficial blood and debris. The solution was changed three times at 3 min intervals with minimal agitation. The tissue was then sonicated in 10 volumes of medium B (0.9% NaCl, 5mM phosphate buffer, pH 7.0). Sonication was for 5 sec in ice at the medium power setting of the MSE 100W disintegrator using an exponentially tapered probe of 3 mm tip diameter. The suspension was removed and the tissue rinsed with a further 10 volumes of medium B. The extracts were pooled and centrifuged at 1000 g for 30 min at 4°C. The supernatant was removed and used directly for some experiments. For other experiments, the supernatant was withdrawn and re-centrifuged at 20,000 g for 40 min at 4°C. The pellet was resuspended in medium B, usually 2 volumes, based on the original weight of the tissue.

The above preparation was designed to minimise the use of organic chemicals, in case substances like organic buffers, proteolytic inhibitors and sucrose stimulate the olfactory receptors. Whole rat brain was used as the control tissue. This was removed, chopped into small pieces, and then prepared in the same way as the olfactory tissue except that the tissue was homogenised instead of sonicated. Protein concentrations

were estimated by using the Hartree variation of the Lowry method[15] using bovine serum albumin as standard. Yields were typically 0.4-0.6 mg of protein per rat in the membrane pellet.

2.2.4 ISOLATION OF CILIA

The rats were killed by cervical dislocation and decapitation. After sagittal section of the head, the turbinates were carefully removed and washed in 5 mM citrate (1.5 ml), pH 6.7, containing 10 mM EDTA, 60 % NaCl, and 0.001 % Triton X-100. Deciliation was by modification of the calcium pulse and 10 % ethanol method[16,17]. The turbinates were stirred in 0.5 ml of deciliation medium (5 mM citrate, pH 6.7, 10 % ethanol, 10 mM CaCl, 3 mM dibucaine), and the cilia were gently brushed off with a paint brush. A further 0.5 ml of the deciliation medium was used to wash off any cilia from the paint brush. The suspension of cilia was centrifuged at 300 g for 20min and the supernatant retained. This cilia preparation was viewed under a scanning electron microscope and assayed for cyclase activity as described below.

2.2.5 SCANNING ELECTRON MICROSCOPY (S.E.M)

50 ul of the above cilia preparation was fixed onto millipore paper, using 2 % glutaraldehyde and 2 % osmium tetroxide as the fixing medium. Dehydration was carried out

in increasing concentrations of tertiary butanol. Samples were fast-frozen and dried overnight in a desiccator containing phosphorus pentoxide. Presence of cilia was investigated using S.E.M.

2.2.6.i Cyclase Assay

The procedure of White and Karr[18] was adopted with minor modifications. This was based on a two-step assay for adenylate and guanylate cyclase using α-^{32}P-labelled ATP or GTP as substrate (summarised in the flow-diagram below, p52). Purification of the resulting ^{32}P-labelled cyclic AMP or cyclic GMP was achieved by sequential chromatography on Dowex 50 and alumina columns. The degradation of the substrate during the assay was minimised by the use of a nucleoside triphosphate generating system, namely creatine phosphate/creatine kinase reaction. Isobutylmethyl-xanthine (IBMX), a phosphodiesterase inhibitor, and the addition of unlabelled cyclic AMP and cyclic GMP were used to minimise product degradation.

The final volume of the reaction mixture was 80 ul and had the following composition: assay buffer (50 mM, pH 7.6, usually phosphate but for some experiments tris-HCl, tris-maleate or arsenate), EGTA (0.12 mM), bovine serum albumin (0.08 mg), IBMX (1.6 mM), phosphocreatine (18 mM), and creatine phosphokinase (8.5 units). The adenylate cyclase reaction mixture contained ATP (1 mM; approx. 300 nCi of [α-^{32}P] ATP),

$MgCl_2$ (2.3 mM), cyclic AMP (3 mM) and GTP (0.01 mM). The guanylate cyclase reaction mixture contained GTP (1 mM;approx. 300 nCi of [α-^{32}P] GTP, $MnCl_2$ (2.3 mM), and cyclic GMP (3 mM). Odorants were added to the above reaction mixture when stimulation of the tissue was required.

Reactions were initiated by the addition of the membrane suspension (25 ul). This was used undiluted for most studies. The tubes were incubated at 30°C usually for 40 min. Reactions were stopped by the addition of 150 ul of 1M perchloric acid ($HClO_4$), and after all the reactions were completed , 300 ul of water containing approximately 10,000 cpm of tritiated cyclic AMP or cyclic GMP was added to each tube . This was in order to estimate recoveries at the column separation stage. After mixing and centrifuging at 500 g for 30 min, the supernatant solutions were poured onto Dowex 50 columns.

2.2.6.ii Column Preparation

Bio-Rad polypropylene econo-column chromatography columns were used. Two perspex racks, capable of holding 72 tubes, were constructed such that one rack may be placed on top of the other. This arrangement considerably accelerated the separation procedure. The alumina columns were prepared by measuring out approximately 1g of alumina and emptying it into a column filled with water. After the alumina had settled and the columns were drained, they were washed with 20 ml of water. Alumina columns were used once only. Dowex 50 columns

were used in the H^+ form. They were cycled before being used, and after each subsequent use, by the sequential addition of one column volume of each of the following: H_2O, 0.5 M NaOH, H_2O, 0.5 M HCl, H_2O.

2.2.6.iii Purification of Cyclic AMP

The Dowex 50 columns contained 1 ml of resin each, which produced a 5 cm bed height. Each column was washed before use with 10 ml of 10 mM $HClO_4$. After the supernatant solution from the rection mixture had drained through the resin bed, 2.5 ml of 10 mM $HClO_4$ was added to each column twice, the first 2.5 ml being allowed to drain through the column before the second was added. All of the column effluent to this point was discarded. The rack of Dowex 50 columns was then mounted above a rack of alumina columns, after which 7 ml of 10 mM $HClO_4$ was added to each Dowex 50 column and allowed to drain through both columns. The Dowex 50 columns were removed and the alumina was washed first with 10 ml of water and then with 1 ml of 0.2 M imidazole buffer.

The alumina columns were then mounted above a rack of scintillation vials and the cyclic AMP was eluted from the alumina and into the vials by the addition of 3 ml of 0.2 M imidazole to each column. After addition of 18 ml of scintillation "cocktail" (8.3 g PPO, 0.33 g POPOP, 667 ml Triton X-100 and 1L Xylene), the vials were counted in a Packard Tri-Carb scintillation counter with the windows set at

optimal values. All results were corrected for quench and for decay of the ^{32}P isotope during the counting process. The counts in the tritium channel allowed for correction of recovery during the column separation which was usually of the order of 50 %.

Results were converted into units of pmoles/min/mg protein or pmoles/min/g tissue using the specific activity of the labelled isotopes after correcting for the decay of the isotope.

2.2.6.iv Purification of Cyclic GMP

The dowex 50 columns for this isolation contained 2 ml of resin giving a 10 cm bed height. Before use, each column was washed with 10 ml of 50 mM HCl. After the supernatant solution from the reaction mixture had drained through the resin, it was followed by the addition of 2.5 ml of 50 mM HCl. This total effluent was discarded. The Dowex 50 columns were then mounted above a rack of alumina columns, and an additional 3.5 ml of 50 mM HCl was added to each Dowex 50 column and allowed to drain through both columns. The remainder of the assay was the same as the cAMP purification described above.

A SUMMARY OF THE CYCLASE ASSAY

Reaction Mixture: 25 ul IBMX +/- Odour (final conc. 1.6mM)
(Total vol 80 ul) 15 ul assay buffer 50 mM pH 7.6
15 ul various nucleotides
Start Reaction --- 25 ul membrane suspension

↓

Incubate 30°C 40 min

↓

Stop Reaction ----- Add 150 ul 1M $HClO_4$

↓

Add300 ul H_2O containing 10,000 cpm [^3H] cAMP
Centrifuge (500 x g, 30 min)

↓

purify supernatant by column chromatography

<u>Purification of cAMP</u>

Pour supernatant onto Dowex column (5cm bed ht.)

↓

Add 2x2.5 ml 10 mM $HClO_4$; discard column effluent

↓

With Dowex columns above alumina columns, add 7 ml 10 mM $HClO_4$ and drain

<u>Purification of cGMP</u>

Pour supernatant onto Dowex column (10 cm bed ht.)

↓

Add 2.5 ml 50 mM HCl; discard column effulent

↓

With Dowex columns above alumina columns, add 3.5 ml 50 mM HCl and drain

Remove Dowex columns and wash alumina columns with 10 ml H_2O and 1 ml 0.2 M imidazole buffer, and drain

↓

add further 3 ml 0.2 M imidazole buffer

↓

Collect effulent in vials, add 18 ml scintillation "cocktail"
Record scintillation counts

2.3 RESULTS

2.3.1 ISOLATION OF CILIA

The aim of this investigation was to isolate a clean preparation of cilia to test for receptor sites. Thus far, the most suitable technique to isolate cilia has been the 10 % ethanol and calcium shock method[16,17], where cilia are detached by modifying the membrane and making it more fragile. The rigid cilia can then be removed by any mechanical shock.

However, SEM studies show that this method is unsuitable for rat olfactory mucosa. The main problems encountered with this technique were: (a) large amounts of mucus covering the surface was retained in the cilia preparation. This needed to be removed without damaging the cilia, (b) olfactory cilia, unlike respiratory cilia, exist in a tangled mass and consequently are more difficult to remove, (c) olfactory cilia, being long, thin and fragile, are prone to breakages and therefore are likely to break at various points and form vesicles. Consequently, what one views under the microscope may not necessarily appear as long and thin strands of cilia. Figure 2.4 shows a typical SEM micrograph obtained with this technique.

Attempts were thus made in this laboratory to modify the 10 % ethanol and calcium shock method. The turbinates were fixed in agarose gel, which was then sliced in an effort to

Fig. 2.4 SEM micrograph of rat olfactory cilia.

detach cilia. This, however, proved difficult and the cilia were impossible to separate from the gel. From further attempts, it was established that reducing the saline concentration in the washing medium helped clear much of the mucus. The use of dibucaine and a paint-brush further facilitated deciliation. This method however gave a very poor yield and S.E.M failed to show the typical 9(2)+2 pattern of cilia.

Assaying for cyclase activity in the cilia preparation initially produced some intriguing results. No adenylate cyclase activity appeared to be present in the preparation. It was later established that ethanol, dibucaine and calcium, used in the deciliation medium, were responsible for this loss in cyclase activity (Table 2.2).

	pmoles cAMP / min./g tissue			
	Untreated	Calcium	Ethanol	Dibucaine
Basal Level	985± 7	170± 14	2020± 8	730± 42
Stimulated Level*	1780± 14	95± 7	1480± 14	505± 8
% Increase	81	-44	-27	-31

* (-)-Carvone (1mM) was used as the stimulant.

Table 2.2 The effect of calcium, ethanol and dibucaine on olfactory adenylate cyclase activity (sonicated preparation). Results are the mean of duplicate determinations with standard deviation.

These chemicals thus needed to be removed before subjecting the samples to the assay procedure. This was achieved by pelletizing the membranes at 30,000 g for 30min, resuspending the pellet, and purifying by sucrose density centrifugation. Active protein was found to be retained between the 40-50 % sucrose density bands. These results correlate well with those from the crude olfactory adenylate cyclase preparation (described below), and thus support the possible involvement of cilia in olfaction. Due to the poor yield of cilia obtained by this method the crude olfactory adenylate cyclase preparation, described below, was then used for further experiments. This preparation ,whilst not yielding recognisable cilia, seems to result in a cyclase activity comparable to the above method and the assumption that most of the cilia are stripped off in this preparation would seem valid. One may therefore speculate that the olfactory receptor proteins, present in the crude olfactory adenylate cyclase preparation are similar to those found in the cilia preparation.

2.3.2 GUANYLATE CYCLASE

Very little guanylate cyclase appeared to be present in olfactory tissue. The amount present was 2-5 pmols/min/mg protein, aproximately one fifth of that found in the brain tissue. Furthermore, guanylate cyclase activity was not affected by the presence of an odour; it can therefore be assumed that this enzyme plays little or no part in olfactory

recognition.

2.3.3 ADENYLATE CYCLASE

The results strongly suggest that the olfactory epithelium is a rich source of adenylate cyclase, comparable to brain membanes. The olfactory preparation yielded adenylate cyclase activity of typically 500 pmol/min/mg protein; boiled olfactory preparation showed no cyclase activity. Homogenates from rat brain gave an adenylate cyclase activity of 20 to 30 pmols/min/mg protein with about 10 times that pelleted by the low speed centrifugation.

An important criterion for a functional role of adenylate cyclase in olfaction would be its modulation by odorants in a cell-free system. Thus the experiments were carried out in the presence of odours that were electrophysiologically proven to be dectectable by the rat olfactory system[19]; these odours were found to enhance the adenylate cyclase activity of the olfactory preparation(Fig. 2.5).

These results also illustrate that the stimulation of adenylate cyclase by odorants depends upon the nature and concentration of the odorant in the incubation mixture, and that the production of cyclic AMP is linear with time under the assay condition. It must be stressed that "no adour" means no added odour; rats themselves posses an odour and there is

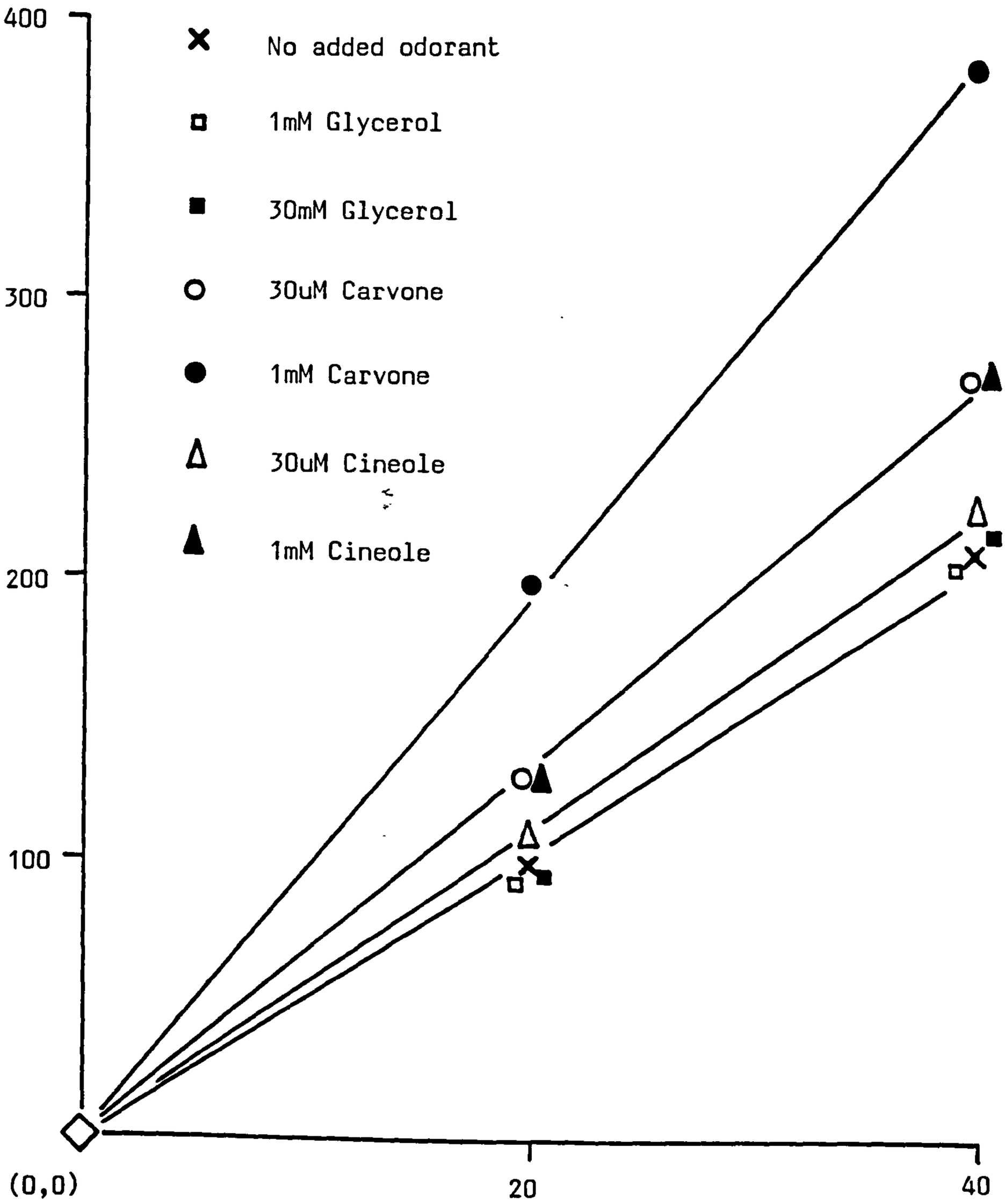


Fig. 2.5 The Effect of Odours and Glycerol on the Olfactory adenylate cyclase.

The supernatant from the low speed centrifugation was assayed without further purification. The olfactory sonicate was incubated for varying times in the presence or absence of odorants. The results are plotted as the mean of duplicate determinations on a single preparation, with a standard deviation of 3%. All odorants gave zero cAMP production at zero time.

no certainty that all small organic chemicals have been removed during the preparation. Of the different odorants included in the reaction mixture, (-)-carvone (1 mM) was found to be the most effective, producing a 100 % increase in basal activity whereas 1-8-cineole (1 mM) produced stimulations of approximately 35 % of the resting activity. Glycerol was used as an example of a non-odorous organic material, failing to stimulate the olfactory cyclase when it was applied at 30 mM (Fig. 2.5). No stimulation of adenylate cyclase activity was observed when 1mM (+)-carvone, (-)-carvone or acetophenone was added to the brain homogenate preparation.

Fig. 2.6 shows that the adenylate cyclase activity increases with the concentration of added odorant. The increase in activity on addition of odorant can be described by the equation;

$$\text{Log } (A) = m \log(c) + \text{constant} \qquad (1)$$

where A is the cyclase activity minus the basal activity, and C is the concentration of the odorant. The same equation is used to describe the variation of the amplitude of the electro-olfactogram (EOG) with odorant concentration[20,21]. The EOG is an indicator of the early electrical events in odour transduction[22]. Receptor heterogeneity[23] could explain the shape of the "enzyme activity" vs "odorant concentration" curve[24]. Polak[24] has suggested that an odorant interacts with more than one receptor and is recognised by the relative degree of binding. Some recent results by Shirley et al.[21], on

the variation of EOG amplitude with odorant concentration, are consistent with this view.

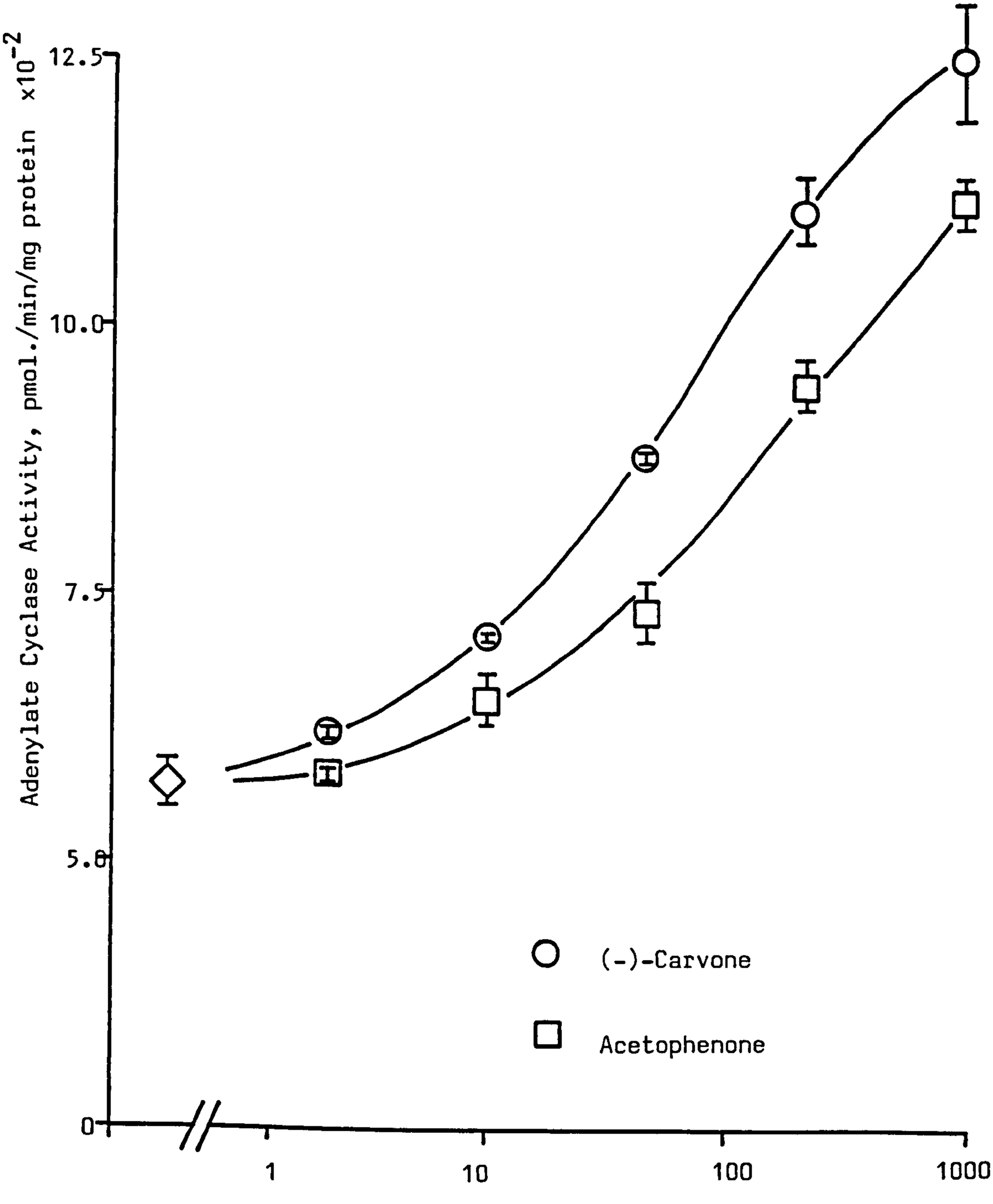


Fig. 2.6 The variation of olfactory adenylate cyclase activity with the concentration of added odorant.

The error bars indicate the standard error mean of triplicate determinations on a single preparation.

Most of the small selection of odorants which were tested, stimulate the adenylate cyclase with variable responses(Fig. 2.7a). At 1mM, (+)-carvone produced nearly the same stimulation as the (-)isomer. 1-8-Cineole, at 1 mM produces stimulation of ca. 35 % of the resting activity. Acetophenone, at 2 mM, shows the highest stimulation. The odorants, citronellol and decanoic acid, however, fail to stimulate. Decanoic acid is predominately ionised in aqueous solution at neutral pH. The sonication procedure adopted in the preparation of the olfactory tissue would be expected to produce membrane vesicles; hence, if the observed cyclase activity originates mainly from everted vesicles, then this odorant may not have free access to the receptors. It is possible that citronellol may have dissolved in the plastic of the reaction tubes. Glycerol, which is non-odorous, also fails to stimulate.

Preliminary experiments (Table 2.3), show that the presence of GTP in the reaction mixture further enhances the odorant effect on adenylate cyclase activity. The table illustrates that the odorant 1-8-cineole, at 1 mM, stimulates the adenylate cyclase activity by ca. 43%. Furthermore, both the stimulated activity as well as the basal activity show an increase of ca. 20% in the presence of 10 uM GTP. This therefore indicates the possible involvement of a G protein, that may be responsible for receptor-cyclase coupling. This possibility is strengthened in further experiments (Fig. 2.7b), where the profound effect of GTP analogues can be seen

Cineole Level	pmoles cAMP / min. g tissue	
	GTP concentration	
	0	10 uM
0	371 ± 1	470 ± 34
1 mM	530 ± 56	620 ± 8

Table 2.3 The effect of GTP and the odorant 1-8-cineole on olfactory adenylate cyclase activity.

The supernatant from the low speed centrifugation was assayed without further purification, in the presence and absence of GTP and an odour. Results are expressed as the mean of duplicate experiments with standard deviation.

to produce a stimulation of approximately 3-4 fold above the basal activity. Sodium fluoride is also seen to stimulate whereas GDβS produces the expected inhibitory effect. These results therefore suggest that receptor and cyclase are linked via a G protein. The level of activity in the absence of added GTP probably indicates that the preparation contains endogenous guanosine nucleotides; the rephosphorylation system present in the assay will maintain these in the form of GTP. Fig.2.7b also illustrates that two substances which might be expected to affect a hormonally contolled cyclase, i.e. histamine (known to increase cyclic AMP levels in brain slices[28]) and isoproterenol (an adrenergic agonist), both fail to stimulate the olfactory adenylate cyclase. This may be an indication that the olfactory cyclase activity is controlled via a non-hormonal pathway.

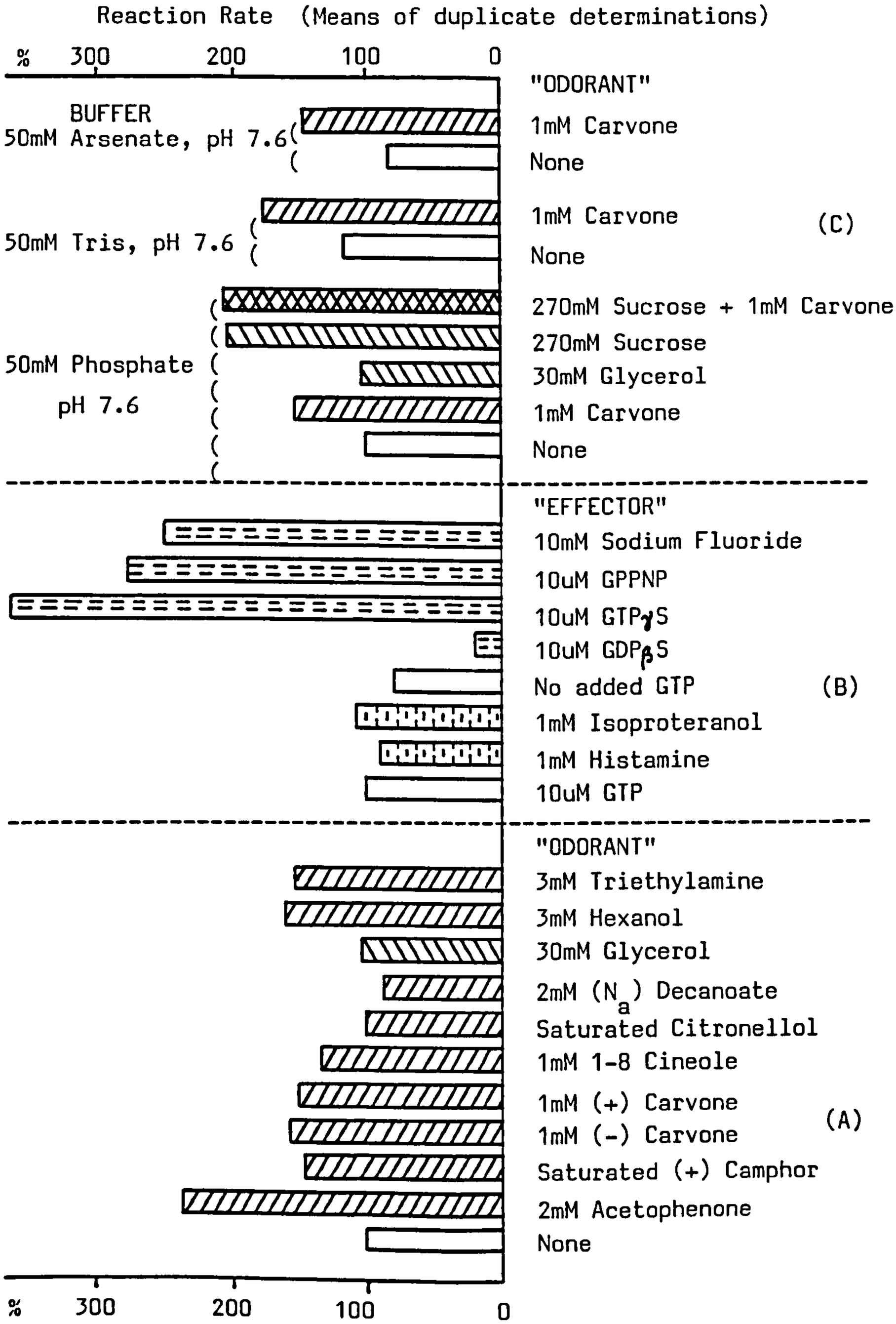


Fig. 2.7 The Olfactory adenylate cyclase activity under a variety of conditions.

The supernatant from the olfactory sonicate was purified by centrifugation (20,000g, 40 min, 4°C). The resuspended pellet was incubated in the presence of various compounds. Parts A, B, and C refer to three different tissue preparations. Throughout the figure the 100% reaction rate is that measured in the presence of 10uM GTP and with no added odorant. The 100% reaction rate for (A) is 520 pmol/min/mg protein, (B) 800 pmol/min/mg protein, and (C) 440 pmol/min/mg protein. Tissue preparation (A) is in phosphate buffer and (B) is in tris-maleate buffer. Standard deviation is 5%.

Adenylate cyclase activity, both basal and odour-stimulated, can be seen irrespective of the type of buffer used in the assay (Fig. 2.7c). Only small differences in activity are observed using different buffers. The buffer showing the greatest ratio of stimulated to basal activity is arsenate (Table 2.4a).

(a)

	pmoles cAMP / min. g tissue		
	Tris-HCl	Phosphate	Arsenate
Basal Level	1090 ± 139	1013 ± 106	827 ± 21
Stimulated Level*	1733 ± 132	1550 ± 126	1500 ± 34
% Increase	59	53	81

(b)

	pmoles cAMP / min. g tissue		
	Tris-HCl	Phosphate	Arsenate
Basal Level	157 ± 57	107 ± 72	120 ± 28
Stimulated Level*	174 ± 49	144 ± 84	77 ± 28
% Increase	11	35	-35

* (-)-carvone (1 mM) was used as the stimulant.

Table 2.4 The effect of various buffers (50 mM, pH 7.6) on olfactory adenylate cyclase activity using (a) a sonicated and (b) a homogenised preparation.

The supernatant from the low speed centrifugation was assayed without further purification, using the said buffers. Results are expressed as the mean of duplicate experiments with standard deviation.

However, this bufer seems to have a deleterious effect: storage of the membranes in arsenate buffer for 18 hours results in an almost total loss of activity. Fig. 2.7c also illustrates that addition of the non-odorant glycerol produces no stimulation. Isotonic sucrose, however, produces a large stimulation and, in the presence of sucrose, no odour-induced stimulation can be observed. Whether this is due to sucrose itself or to some "odorous" impurity in the sucrose has not been investigated.

Most membrane preparations involve homogenisation of the tissue. Olfactory tissue was subjected to both homogenisation and sonication (Tables 2.4a,b). The results show that when a sonicated preparation is used, (-)-carvone (1 mM) increases the adenylate cyclase activity by ca. 60%. However, on using a homogenised preparation, the same odorant produces a stimulation of only ca. 10%. The results therefore favour sonication; homogenisation appears to destroy cyclase activity.

Further purification of the crude olfactory preparation was obtained by centrifuging at 30,000 g for 30min. Most of the cyclase activity was found to be present in the pellet, very little being retained in the supernatant (Fig. 2.8). The supernatant from a 300 g, 30min centrifugation of the crude sonicate was subjected to sequential 30min centrifugations at the indicated speeds. Each of the five pellets showed

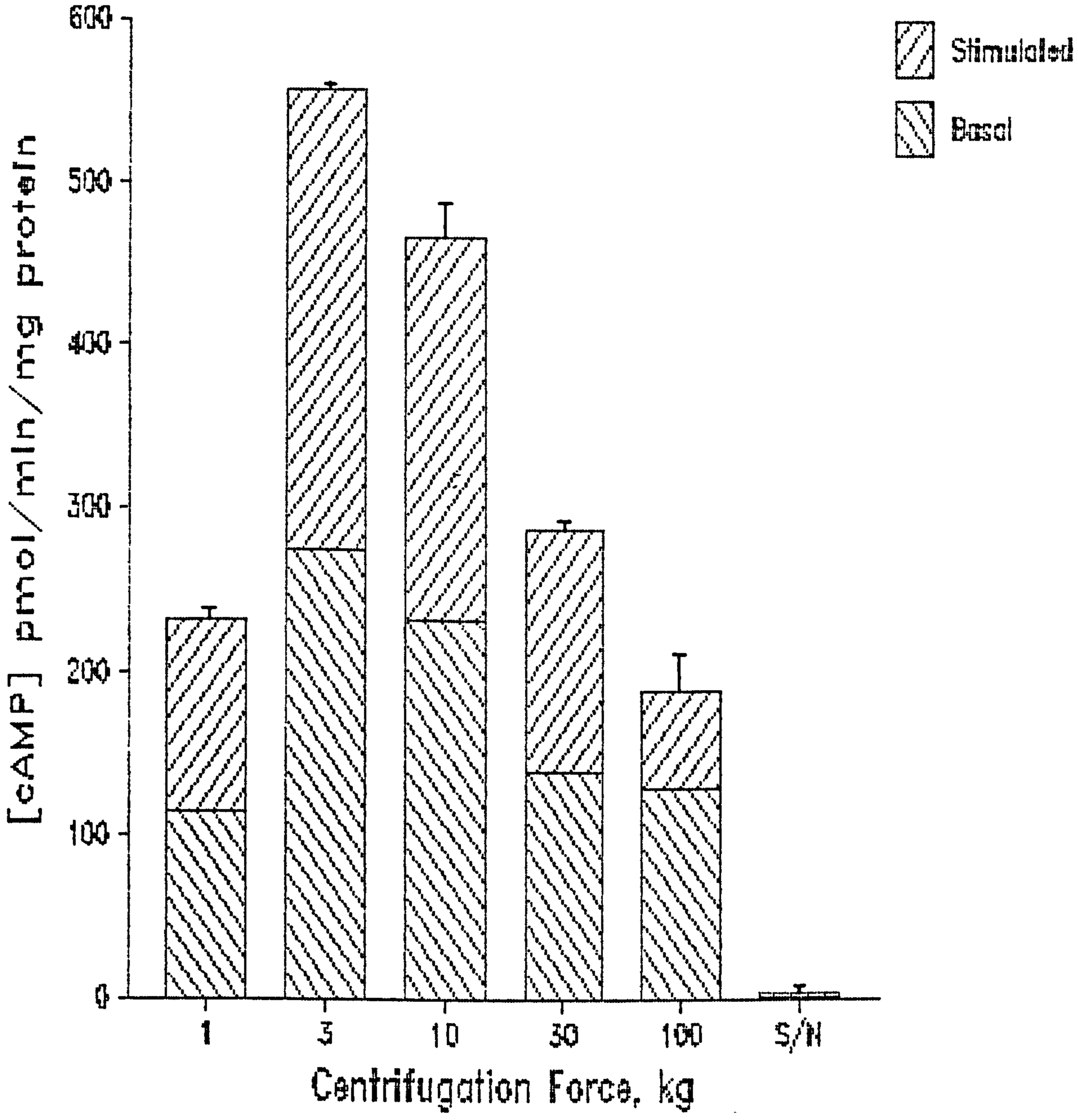


Fig. 2.8 The effect of different degrees of centrifugation on olfactory adenylate cyclase activity.
The supernatant from the low speed centrifugation of the olfactory sonicate was subjected to sequential 30 min centrifugation at the indicated speeds. Each of the five pellets thus formed, showed adenylate cyclase activity. On addition of 1mM (-)-carvone, each pellet showed an increase in activity. Very little activity was found in the final supernatant. Results are the means of duplicate determinations with standard deviations.

adenylate cyclase activity. On addition of 1 mM (-)-carvone, each showed an increase in activity ranging from 100 % to 108% for the 1000 g to 30,000 g pellets, and 47 % for the 100,000 g pellet. The total recovery of starting activity was 89 % and of this 60 % appeared in the 3,000 g and 10,000 g pellets, which also showed the highest specific activity. Less than 4 % of the recovered activity was found in the final supernatant. The adenylate cyclase activity is therefore associated only with the particulate fraction of the sonicate and, as indicated by Fig. 2.8, the stimulable component co-sediments with the basal.

The effect of storing olfactory preparations, over periods of several weeks at -20°C, on adenylate cyclase activity was investigated (Table 2.5), since the tissue preparation procedures are long and laborious.

	pmoles cAMP / min. g tissue	
	after freeze/thaw	before freeze/thaw
Basal Level	1010 ± 132	915 ± 106
Stimulated Level*	2127 ± 135	1812 ± 105
% Increase	111	98

* (-)-carvone (1mM) was used as the stimulant.

Table 2.5 The effect of sample-freezing on olfactory adenylate cyclase activity (sonicated preparation).

Results are expressed as the mean of duplicate determinations with standard deviation.

No apparent loss in cyclase activity was detected. The membranes can therefore be prepared well before the experiment and stored at -20°C in phosphate buffer. A slight increase in cyclase activity was usually apparent on freezing and thawing.

Fig. 2.9 illustrates the deleterious effect of calcium when present in the assay medium. Calcium is seen to inhibit both odour-stimulated and basal adenylate cyclase activity. The addition of EGTA results in an increase of activity of both components. Further experiments by Shirley and Robinson[28], using calcium buffers, showed that inhibition of the cyclase activity occurs at calcium concentrations at and above the physiological range 10^{-8} M to 10^{-6} M.

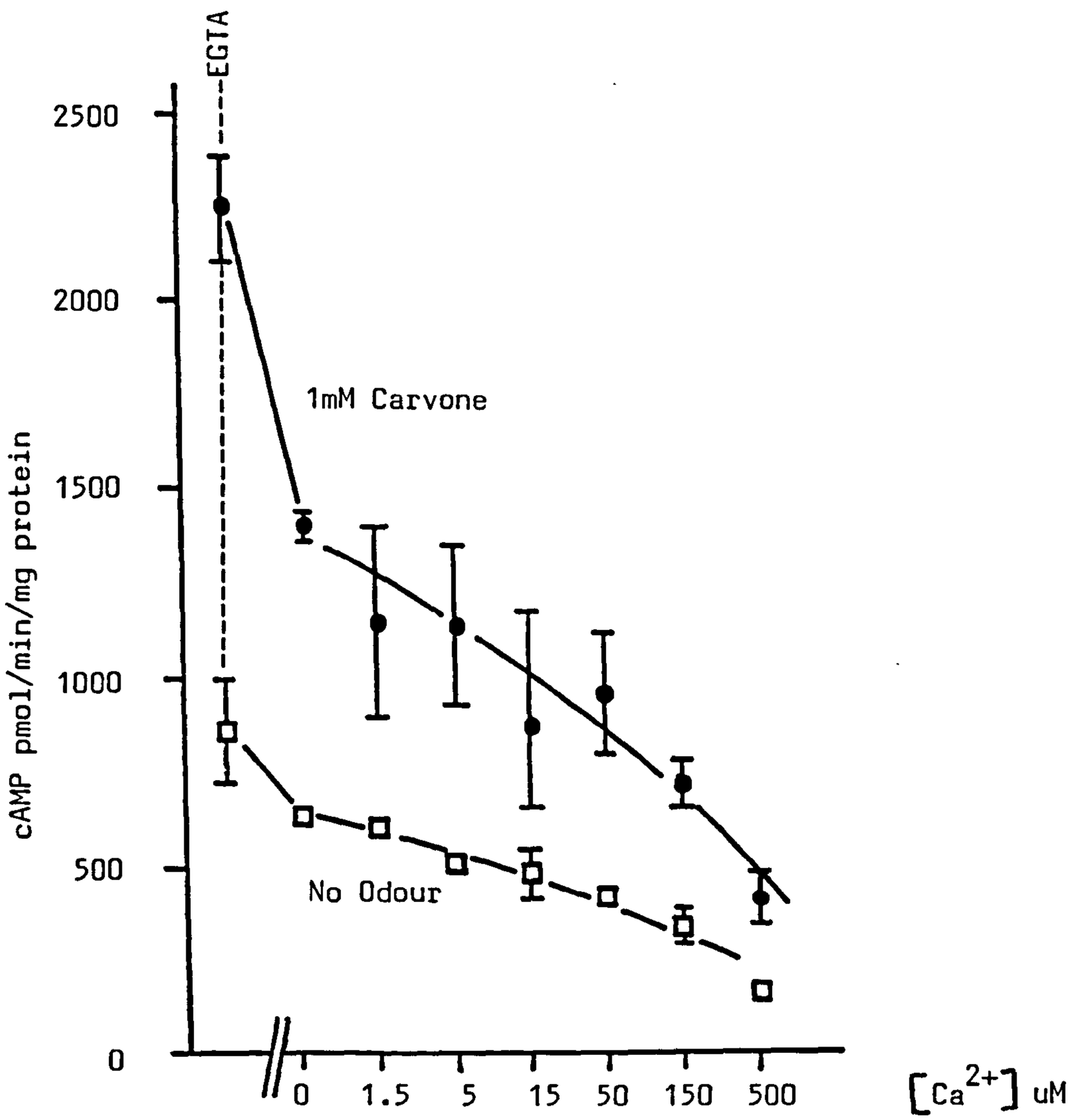


Fig. 2.9 The effect of calcium concentration on olfactory adenylate cyclase activity.

The supernatant from the low speed centrifugation was used without further purification. The olfactory sonicate was incubated in the presence of various concentrations of added calcium or 100uM EGTA. The effects on both basal (no added odour) and stimulated (1mM (-) Carvone) activity were investigated. The results are the mean of duplicate determinations with standard deviation.

2.4 DISCUSSION

The experimental results provide good evidence that the rat olfactory mucosa contains an adenylate cyclase which is sensitive to physiological concentrations of odorants. The activity varies with concentration of odorant in a way which parallels the early electrical events of odour transduction. There seems to be no hormonally-controlled component to the cyclase activity. This evidence leads to the conclusion that rat olfaction is mediated by a cyclic AMP system. This has also been found in the frog[23].

The odorant concentrations necessary to stimulate adenylate cyclase <u>in-vitro</u> correlates well with those known to give measureable <u>in-vivo</u> summated electrophysiological recordings. Such recordings are typically carried out at 10^{-2} to 10^{0} vapour saturation[27] for odorous compounds, respectively having vapour pressures of 5.0 - 0.05 mm Hg (at 25°C). Then, as Pace and Lancet[23] suggest, with molar water/air partition coefficients being in the range of 1-1000, the physiological aqueous odorant concentrations would be uM - mM which are in good agreement with the experimental results.

Kurihara and Koyama[12] in 1972, did not test the adenylate cyclase activity of their olfactory membrane preparations for stimulation by odorants. Menevse et al.[13], in 1977, found that odorants did not stimulate the cyclase activity of their membranes . These membranes were prepared by

the method of Koch[28], i.e. in sucrose (Dodd, G.H. - personal communication). The results reported here show that sucrose stimulates the cyclase to the point where odour stimulation is masked, and therefore would probably explain the apparent unresponsiveness of these membranes. Another possible reason for this apparent lack of stimulation could be the homogenised olfactory preparation that Menevse[29] used in his investigation; this study has shown that homogenisation decreases cyclase activity (Table 2.4).

The olfactory transduction mechanism is believed to reside in the cilia and terminal swellings of the olfactory primary cells[30]. Direct verification of the role of cilia in odorant recognition could be accomplished with an isolated funtionally active cilia preparation. Such a preparation would also allow detailed examination of the specificity and mechanisms of odorant-cilia interactions. However, the calcium-shock method of cilia detachment used in this report gave a very poor yield. Sklar et al.[31] who have subsequently confirmed the presence of an odorant-stimulated adenylate cyclase in the rat, use this method of calcium-shock in order to obtain a cilia preparation but fail to give any estimate of the yield. A poor yield of cilia from rat olfactory epithelium was also reported by Pace and Lancet[32]. Due to the apparent unreliability of this preparation to provide a reasonable yield of cilia the subsequent experiments were performed using the crude olfactory adenylate cyclase, described above, with

success.

Caution is necessary when comparing levels of activity of an enzyme prepared from different methods. However, the olfactory preparation obtained in this study, shows higher activity than brain membranes prepared in the same buffer. The activity of the olfactory material is typically 500 pmol/min/mg protein which is about the same as that of brain membranes prepared in more conventional buffer systems, typically 500 pmol/min/mg protein[33]. The brain is generally believed to be the tissue containing the highest levels of adenylate cyclase, but it seems reasonable to conclude that the olfactory levels are at least comparable.

Pace et al.[23] report a basal cyclase activity of approximately 5-10 nmol/min/mg protein which is about ten to twenty times that found in the present investigation. However, Pace et al. assayed protein by the method of Bradford[34] which detects lower values than measured by the method of Lowry[15], using bovine serum albumin as standard. Sklar et al.[31], using the Lowry method of protein estimation, report basal adenylate cyclase activities of about 2 nmol/min/mg protein in the frog olfactory cilia, and 3-4 nmol/min/mg protein in the rat olfactory cilia. The slight discrepancies in results are probably due to differences in assay conditions.

The results of the experiments with guanosine nucleotide analogues strongly suggest that the receptor and cyclase are

linked via a G protein[3]. This linkage and the sensitivity of the cyclase to calcium are further points of similarity between rat and frog.

The dependence of olfactory adenylate cyclase activity on guanine nucleotides suggests that a signal-transducing G protein is involved, similar to the hormonal G protein[3] and to visual transducin[35]. Similar observations have been made by Pace et al.[23] and Sklar et al.[31]. These observations together with the fact that odorants and GTPγS increase rather than decrease ciliary adenylate cyclase suggest that the G_s-like protein is the specific neuronal membrane component responsible for receptor-cyclase coupling. Furthermore, Pace and Lancet[32] and Anholt et al.[36], have recently established the presence of high concentrations of the the G protein G_s in the frog, toad and rat olfactory epithelia. The presence of G_i and G_o were also confirmed. G_i was found to be present in minor quantities whereas substantial amounts of G_o were measured in the olfactory cilia. It is thought possible that G_o may be linked with phosphatidylinositol turnover[7] or with potassium channels[11].

An interesting corroboration of the possible role of G_s in olfaction comes from two independent studies carried out in other laboratories. Patients with pseudohypoparathyroidism, a genetic disease that involves resistance to parathyroid and other hormones, have been reported to have impaired olfactory sensitivity[37]. It has been independently demonstrated that

pseudohypoparathyroidism, at least in some patients is accompanied by a G_s deficiency[38]. Together, these results are consistent with the involvement of G_s in olfactory transduction.

The fact that calcium has an inibitory effect on both basal and stimulated adenylate cyclase activity implies that it may be involved in the regulation of the system. The olfactory mucosa is known to contain high levels of adenylate phosphodiesterase whose iso-enzyme pattern changes with neuronal degeneration[39]. Other workers in this laboratory have recently investigated this enzyme and found it to be stimulated by calcium (Keith Dickenson - personal communication). An increase of calcium following stimulation of the olfactory tissue would therefore serve to return the system to its resting level by stimulating the phosphodiesterase and consequently inhibiting the adenylate cyclase. It is thus possible that the second messenger in olfaction has a closed loop structure, as is believed to be the case for most second messenger systems[40]. For instance, an odorant binding to a receptor stimulates cyclase activity; as a result cyclic AMP levels rise causing the sodium ion gates to open; this in turn depolarises the membrane followed by release of calcium; the released calcium inhibits the cyclase activity and stimulates the phosphodiesterase, thereby completing the cycle. The inhibition of olfactory adenylate cyclase activity by calcium has also been confirmed by Sklar et al.[31].

Another important receptor-couple effector system is phosphatidylinositol turnover (see Chapter 4). In response to receptor activation phospholipase C degrades phosphatidyl-inositol 4,5-bisphosphate, a phospholipid, to form dual second messengers. One messenger, inositol trisphosphate (IP_3), liberates calcium ions from internal stores; the other, diacylglycerol stimulates protein kinase C, that, like the cyclic AMP-dependent enzyme, phosphorylates multiple protein substrates. It is not yet known if receptor-coupled phosphoinositide break-down is present in olfaction, but if so, an interaction between the two major signalling pathways is possible. The release of calcium caused by IP_3 has the potential to modulate effects of cyclic AMP by interacting with the phosphodiesterase and adenylate cyclase. Furthermore, it is of interest to note that Anholt et al.[30] have recently identified protein kinase C in olfactory cilia. This protein kinase C however was also located in the respiratory epithelium.

Is adenylate cyclase activation the only mechanism for olfactory transduction? Sklar et al.[31] investigating a series of odorants found that many odorants (notably those in the fruity, minty and herbaceous classes), activate adenylate cyclase, but other physiologically active odorants (e.g. putrid aliphatic acids, amines and organic solvents) do not affect the enzyme in isolated cilia. Thus, it is possible that certain olfactory receptor molecules may generate an intracellular signal through different transduction

mechanisms. The failure of citronellol and decanoic acid to stimulate the cyclase in this report may be explained by the possible presence of an alternative transduction mechanism. Another explanation, however, would be that in aqueous solution at neutral pH, decanoic acid is predominantly ionised. If a large fraction of the observed cyclase activity derives from everted vesicles, then the decanoate ion would reach the receptor only with difficulty and so appear to be a poor stimulus, while most uncharged odorants should cross the membrane fairly easily. Triethylamine, another "ionic" odorant was only a weak stimulus. Citronellol may have dissolved in the plastic of the reaction tubes. Solution of odorants into plastic and adsorption onto glass are major technical problems encountered in this kind of experiment.

If odorants act through a second messenger, then certain criteria of nucleotide involvement in this physiological process must be fulfilled , as laid down by Sutherland[1]. For the olfactory system these criteria[13] are as follows:

(1) The receptor cells should contain adenylate cyclase and the odorants should alter the intracellular cyclic AMP levels in these cells.

(2) Odorants should modulate the activity of adenylate cyclase in a plasma membrane preparation from the receptor cells.

(3) Inhibitors of the phosphodiesterase which destroys the cyclic AMP should affect the action of the odorants on the

olfactory mucosa.

(4) Cyclic AMP or its derivatives should be able to stimulate the action of odorants on the tissue.

The experiments reported in this study investigate the second of the Sutherland criteria and provide good evidence for the existence of an odorant-stimulated adenylate cyclase in the rat. This second criterion has also been satisfied by Pace et al.[23] in the frog and Sklar et al.[31] in the frog and the rat. The third and fourth criteria were investigated by Minor and Sakina[41] and Menevse et al.[13]. These workers showed that cyclic AMP analogues and phosphodiesterase inhibitors affected the production of the EOG, therefore providing strong evidence for the specific involvement of cyclic AMP in the production of olfactory generator potentials.

Attempts have also been made in this laboratory to investigate the first Sutherland criterion (see Chapter3).

It can thus be concluded that these results, together with the previously reported <u>in-vivo</u> modulation of olfactory responses by cyclic AMP analogues and phosphodiesterase inhibitors, provide most of the criteria necessary for establishing the role of cyclic AMP as a second messenger in olfactory reception. Measurement of odorant-stimulated adenylate cyclase in olfactory cilia provides the first <u>in-vitro</u> assay for olfactory reactivity, and could complement

electrophysiological recordings in the study of odorant recognition. The olfactory adenylate cyclase should be a powerful tool for the study of olfactory receptors in-vitro providing a monitor for the solubilisation and purification of the receptors. Olfaction appears to bear a striking molecular similarity to hormone, neurotransmitter and visual reception.

REFERENCES

1. Robinson, G.A., Butcher, R.W. and Sutherland, E.W. (1971) "Cyclic AMP" (Academic Press, New York-London), pp. 531.

2. Stryer, L. (1986) Annu. Rev. Neurosci., 9, 87-119.

3. Gilman, A.G. (1984) Cell, 36, 577-579.

4. Northup, J.K. (1985) "Molecular Aspects of Cellular Regulation", eds. Cohen, P. and Hanslay, M.D. (Elsevier Biochemical Press), Vol. 4, pp. 91-116.

5. Fung, B. (1985) "Molecular Aspects of Cellular Regulation", eds. Cohen, P. and Hanslay, M.D. (Elsevier Biochemical Press), Vol. 4, pp. 183-214.

6. Sternweis, P.C. and Robinshaw, J.D. (1984) J. Biol. Chem., 259, 13806-13813.

7. Cockcroft, S. and Gomperts, B.D. (1985) Nature (London), 314, 534-536.

8. Enjalbert, A., Sladeczek, F., Guillon, G., Bertrand, P., Shu, C., Epelbaum, J., Garcia-Sainz, A., Jard, S., Lombart, C., Kordon, C., and Bockaert, J. (1986) J. Biol. Chem., 261, 4071-4075.

9. Heyworth, C.M., Whetton, A.D., Wong, S., Martin, B.R., and Houslay, M.D. (1985) Biochem. J., 228, 593-603.

10. Wakelam, M.J.O., Davies, S.A., Hanslay, M.D., McKay, I., Marshall, C.J., and Hall, A. (1986) Nature (London), 323, 173-176.

11. Pfaffinger, P.J., Martin, J.M., Hunter, D.D., Nathanson, N.M., Hille, B. (1985) Nature, 317, 536-538.

12. Kurihara, K., and Koyama, N. (1972) Biochem. Biophys. Res. Commun., 48, 30-34.

13. Menevse, A., Dodd, G. and Poynder, T.M. (1977) Biochem. Biophys. Res. Commun., 77, 671-677.

14. Chen, Z. and Lancet, D. (1984) Proc. Natl. Acad. Sci.,81, 1859-1863.

15. Hartree, E.F. (1972) Anal. Biochem., 48, 422-427.

16. Linck, R.W. (1973) J. Cell Sci., 12, 345-367.

17. Rhein, L.D. and Cagan, R.H. (1980) Proc. Natl. Acad. Sci. USA, 77, 4412-4416.

18. White, A.A. and Karr, D.B. (1978) Anal. Biochem., 85, 451-460.

19. Shirley, S.G., Polak, E.H. and Dodd, G.H. (1983) Eur. J. Biochem., 132, 485-494.

20. Ottoson, D. (1956) Acta. Physiol. Scand., 35, Suppl. 122, 1-38.

21. Shirley, S.G., Polak, E.H., Edwards, D.A., Wood, M.A., and Dodd, G.H. (1987) Biochem. J., 245, 185-189.

22. Ottoson, D. (1970) "Taste and Smell in Vertebrates", eds. Wolstenholme, G.E.W. and Knight, J. (J. and A. Churchill, London), pp. 343-356.

23. Pace, U., Hanski, E., Saloman, Y., and Lancet, D. (1985) Nature (London), 316, 255-258.

24. Polak, E. (1973) J. Theor. Biol., 40, 469-484.

25. Nathanson, J.A. (1977) Phys. Rev., 57, 157-256.

26. Shirley, S.G., Robinson, C.J., Dickenson, K., Aujla, R., and Dodd, G.H. (1986) Biochem. J., 240, 605-607.

27. Ottoson, D. (1971) "Handbook of Sensory Physiology, Vol. 4)", ed. Peidler, L.M. (Springer, Berlin), pp. 95-131.

28. Koch, R.B. (1969) J. Neurochem., 16, 145-157.

29. Menevse, A. (1976) Ph.D. Thesis, Univ. of Warwick.

30. Getchell, T.V., Margolis, F.L., Getchell, M.L. (1984) Prog. Neurobiol., 23, 317-345.

31. Sklar, P.B., Anholt, R.R.H., and Snyder, S.H. (1986) J. Biol. Chem., 261, 15538-15543.

32. Pace, U., and Lancet, D. (1986) Proc. Natl. Acad. Sci. USA, 83, 4947-4951.

33. Johnson, R.A., and Sutherland, E.W. (1974) Meth. Enz., 38, 135-143.

34. Bradford, M.M. (1976) Anal. Biochem., 72, 248-254.

35. Stryer, L., Hurley, J.B., and Fung, K.K. (1981) Trends Biochem. Sci., 6, 245-247.

36. Anholt, R.R.H., Mumby, S.M., Stoffers, D.A., Girard, P.R., Kuo, J.F., and Snyder, S.H. (1987) Biochem., 1987, 26, 788-795.

37. Weinstock, R.S., Wright, H.N., Spieget, A.M., Levine, M.A., and Moses, A.M. (1986) Nature, 322, 635-636.

38. Farfel, Z., Brothers, V.M., Brickman, A.S., Conte, S., Neer, R., and Bourne, H.R. (1981) Proc. Natl. Acad. Sci. USA, 78, 3098-3102.

39. Margolis, F.L. (1975) "Society for Neuroscience Symp., Vol. 3", eds. Perrendelli, J.A. (Bethesda), pp. 167-188.

40. Rasmussen, H., and Goodman, D.B.P. (1977) Physiol. Rev., 57, 3, 421-509.

41. Minor, A.V., and Sakina, N.L. (1973) Neirofiziologiya, 5, 415-422.

CHAPTER 3

INTRACELLULAR LEVELS OF CYCLIC AMP IN OLFACTORY TISSUE

3.1 INTRODUCTION

3.1.1 CYCLIC AMP

Cyclic AMP is recognised as a versatile regulatory agent which acts to control the rate of a number of cellular processes (as described in Chapter 2). It occurs in all animal species investigated, including bacteria and other unicellular organisms. Table 3.1 lists some of the Mammalian body fluids in which the levels of cyclic AMP have been measured. The concentration of cyclic AMP in plasma, CSF and gastric juice is normally quite low, of the order of 10^{-8} M, but its concentration in milk and urine is relatively high, ca. 10^{-6} M

Fluid	cyclic AMP Level (approx.)
Plasma	2 x 10-8 M
Cerebrospinal Fluid	2 x 10-8 M
Gastric Juice	2 x 10-8 M
Milk	10-6 M
Urine	10-6 M

Data taken from Robinson et al.[1], p29.

Table 3.1 Mammalian body fluids in which the level of cyclic AMP has been measured.

The concentration of many cell constituents (e.g. ATP) remain fairly stable under normal physiological conditions. The concentration of cyclic AMP, however, differs in that it is subject to large fluctuations due to factors such as the effect of hormones. Glucagon, for example, is capable of increasing the cyclic AMP concentration in the isolated perfused rat liver by 80 times[2]. The effect of ACTH on the cyclic AMP in the adrenal cortex is even more striking. However, the changes in the cyclic AMP level which are physiologically important are often small compared to the changes that can occur under certain experimental conditions. Hence, although high concentrations of glucagon are capable of causing an 80-fold increase in the hepatic cyclic AMP level, physiological concentrations may produce only a 3-fold increase.

In many tissues the intracellular level of cyclic AMP is one of the most important and also one of the most sensitive factors regulating cell function. Small changes in the cyclic AMP level may lead to effects which are very large relative to the functional capacity of the affected cells. These changes may occur in both directions, an increase above the baseline leading to one effect and a decrease below the baseline leading to the opposite effect. Sometimes, these changes in the cyclic AMP level occur in response to different hormones. In the liver, for example, glucagon tends to increase the level of cyclic AMP while insulin tends to lower it. Many hepatic functions reflect a subtle balance between the

opposing actions of these two hormones[3]. In certain other cases opposite changes in the level of cyclic AMP may occur in response to the same hormone acting under different conditions. Epinephrine, for example, is capable of interacting in some tissues with two different types of receptors. Interaction with one type leads to an increase in the level of cyclic AMP while interaction with the other leads to a decrease, and the net effect of epinephrine in these tissues will depend on whichever type of receptor predominates[3].

In most cases, the intracellular concentration of cyclic AMP at any given instant will depend primarily on the relative activities of the enzymes adenylate cyclase and phosphodiesterase. The relative activities of these enzymes are influenced by a number of factors in addition to the effect of hormones (see Chapter 2). The cyclic AMP concentration might therefore be expected to vary somewhat from one tissue to another, but in general, in the absence of stimulation by exogenous hormones the concentration has been found to be similar in a variety of tissues (between 0.5 to 2.6 pmoles/mg protein). However, the actual concentration of free cyclic AMP may be much less due to, for example, protein binding. It is also possible that in certain parts of some cells it may be much higher as a result of compartmentalisation. The subcellular distribution of cyclic AMP is in need of further study.

3.1.2 AIMS

The aim of this thesis has been to achieve an understanding of the molecular mechanisms that may be involved in olfactory transduction. In the previous chapter (Chapter 2) the rat olfactory epithelium was found to contain high levels of adenylate cyclase. Its activity was enhanced in the presence of odours. The experiments reported therein fulfil the second Sutherland criterion[1], laid down to verify the involvement of a nucleotide in a physiological process. This criterion states that odorants should modulate the adenylate cyclase activity in a plasma membrane preparation of the receptor cells. Various workers[4,5] have since also demonstrated that odorants enhance adenylate cyclase activity. There is much evidence that odorants act through the second messenger, cyclic AMP.

Here, an attempt is made to fulfil the first Sutherland criterion, i.e. odorants should alter the intracellular cyclic AMP levels in the receptor cells.

3.2 METHODOLOGY

3.2.1 ANIMALS

Male wistar rats about 300g body weight were used. The animals were allowed food and water prior to the experiments.

3.2.2 MATERIALS

[8-^{3}H] cyclic AMP, and [adenine-4-^{14}C] cyclic AMP were obtaind from Amersham International. All other biochemicals were purchased from Sigma Chemical Co. and were of the highest purity commercially available. Odorants were obtained from the Aldrich Chemical Company Ltd., and were of the highest quality. Cellulose ester Millipore filters (cat. no. HAWP025) were supplied by Millipore. Diethylether, obtained from Fisons, was of analytical grade.

3.2.3 TISSUE PREPARATION

The animals were killed by cervical dislocation and decapitation. After sagittal sectioning of the head the ethmoturbinates were carefully removed and rapidly fixed by compressing (freeze-clamping) between two copper discs (2.5cm dia.) welded onto a pair of tongs (pre-cooled in liquid nitrogen). The flattened tissue was immediately powdered using a pestle and mortar (also pre-cooled in liqiud nitrogen). The powder was then transferred into a tube containing ice-cold trichloroacetic acid, for cyclic AMP extraction. In some experiments the turbinates were not powdered following freeze-clamping, whereas in others, they were neither freeze-clamped nor powdered prior to the cyclic AMP extraction.

3.2.4 ODOUR STIMULATION

In order to stimulate the tissue, a set of turbinates from one half of the rat head were exposed to an odorant vapour by suspending the tissue in the head-space above the liquid odorant contained in a vial. The stimulation was carried out for different lengths of time. The remaining set of turbinates from the other half of the rat head was used as a control and was held in the air for the same periods of time. At the end of the stimulation period, the tissue was rapidly freeze-clamped and powdered as described above.

In some experiments, liquid odorant stimulation, rather than the vapour stimulation, was used where the freshly dissected turbinates were immersed in the odorant solution for various periods of time. Whole brain tissue was used as a control to verify the effect of an odorant on the olfactory turbinates.

3.2.5 EXTRACTION OF CYCLIC AMP

The extraction technique used is a modification of the method adopted by Mayer et al[5]. The appropriately prepared tissue, described above, was rapidly transferred into a tube containing 2 ml of ice-cold 10 % v/v trichloroacetic acid and immediately sonicated on ice. The sonication was performed for 3 x 5 sec at the medium power setting of the M.S.E. 100 W disintegrator, using an exponentially tapered probe of 3 mm

tip diameter. The sample was cooled on ice for a period of 30 sec between each sonication.

After allowing the specimen to stand for 10 min on ice, it was centrifuged at 3,000 x g, at 3°C, for 20 min. The supernatant was removed and transferred into a glass tube. The trichloroacetic acid was removed from the sample by three sequential washes with two volumes of diethylether. After each wash, the top ether layer was removed and discarded, the cyclic AMP remaining in the lower aqueous layer. After the last ether extraction, the tube containing the aqueous solution was placed in a boiling water bath for two minutes to remove the residual ether. The neutralised extract was then stored at -20°C for cyclic AMP assaying.

The pellet obtained after centrifugation was assayed for protein concentration, by using the Hartree variation of the Lowry method[7]. Bovine serum albumin was used as the standard. Prior to the protein assay, trichloroacetic acid was removed by the addition of one volume of diethylether and the neutralised pellet was resuspended in 2 N NaOH solution.

3.2.6 CYCLIC AMP ASSAY

The method of Gilman[8] was adopted with minor modifications (see flow diagram, p87). The final volume of the reaction mixture was 50 ul and consisted of sodium acetate (500 uM, pH 4.0), [^{3}H] cyclic AMP (1 pmole) and inhibitor

protein (15 ug). Standard cyclic AMP, typically 0.20 pmoles, varying aliquots of unknowns, or appropriate diluents to volume were then added. The tubes were then placed in an ice-bath and the reactions initiated by adding 15 ul (40 ug/ml) of the binding protein (cyclic AMP dependent protein kinase C). The binding protein was diluted prior to use with bovine serum

SUMMARY OF CYCLIC AMP ASSAY

Reaction Mixture: (Total 50 ul)
- 5 ul sodium acetate (500 uM, pH 4.0)
- 5 ul [^{3}H] cAMP (1pmole)
- 5 ul Inhibitor protein (15 ug)
- 20 ul Standard cAMP (0-20 plomes)

↓

Ice bath

Start Reaction ---- Add 15 ul binding protein (40 ug/ml)

↓

Incubate 0°C, 1hr

↓

Stop Reaction------ Add 1 ml KH_2PO_4 (20 mM, pH 6.0)

↓

Filter mixture under vacuum

↓

Place filters in counting vials
Dissolve in 1 ml methyl cellosolve
Add scintillation cocktail (10 ml) and count

albumin such that 15 ug of albumin was added to each assay tube.

The reaction tubes were allowed to incubate at 0°C for one hour, by which time an equilibrium has been shown to be

established[e]. The reactions were terminated by the addition of potassium phosphate (1 ml, 20 mM, pH 6.0), at 0°C, and the solution was gently filtered through 25 mm cellulose ester Millipore filters, pre-rinsed with the same buffer. The filter was immediately washed with 2 x 5 ml of the buffer to remove the unbound [^{3}H] cyclic AMP. Finally, the filters were placed in counting vials containing 1 ml of methyl cellosolve, in which they readily dissolve, and a scintillation cocktail (10 ml) of toluene : methly cellosolve (3:1, v/v). PPO (0.4 % w/v) and POPOP(0.01 % w/v) were added as fluors. The vials were counted in a Packard Tri-carb scintillation counter. All results were corrected for quench and converted to pmoles cAMP/mg protein.

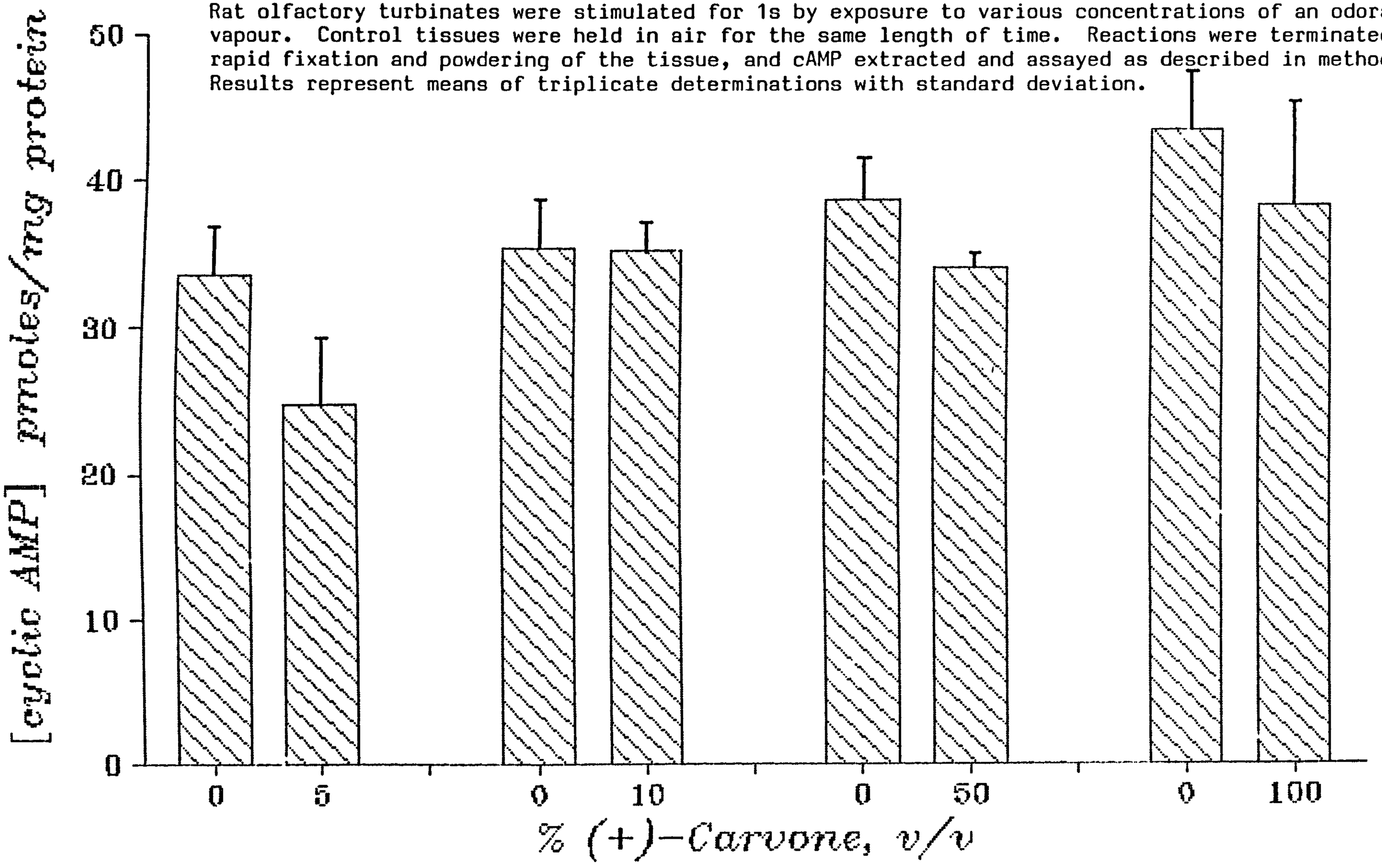
3.3 RESULTS

The olfactory tissue was found to contain high levels of intracellular cyclic AMP (ca.40 pmoles cyclic AMP/mg protein), almost twice as that found in the brain tissue. However, these levels remained unaffected in the presence of an odour (see Fig. 3.2).

The intracellular cyclic AMP levels were measured using the cyclic AMP assay described above, which allows the measurement of low cyclic AMP concentrations (ca. 0.05 pmole). Fig. 3.1 shows typical calibration curves obtained for this cyclic AMP assay. The assay is based on the competition between unlabelled cyclic AMP and a fixed quantity of the

Fig. 3.2 The effect of an odour ((+)-carvone) on intracellular cyclic AMP levels of the rat olfactory tissue.

Rat olfactory turbinates were stimulated for 1s by exposure to various concentrations of an odorant vapour. Control tissues were held in air for the same length of time. Reactions were terminated by rapid fixation and powdering of the tissue, and cAMP extracted and assayed as described in methodology. Results represent means of triplicate determinations with standard deviation.



tritium labelled nucleotide for binding to a protein (cyclic AMP-dependent protein kinase C). The protein has a high affinity and specifity for the cyclic AMP[a]. The heat stable protein-inhibitor of the protein kinase and high concentrations of BSA were used to increase the affinity of the kinase for the cyclic nucleotide[a]; the binding protein's capacity being optimal at pH 4.0[a].

The amount of labelled protein-cyclic AMP complex formed is non-linearly and inversely related to the amount of unlabelled cyclic AMP present in the assay sample (Fig. 3.2a). Measurement of the protein-bound radioactivity enables the amount of unlabelled cyclic AMP in the sample to be calculated. A linear relationship may be obtained by plotting C_o/C_x against pmoles of standard cyclic AMP (see Fig. 3.2b), where C_o is the cpm bound in the absence of unlabelled cyclic AMP (i.e. the zero-dose binding) and C_x is the cpm bound in the presence of unlabelled cyclic AMP. The amount of cyclic AMP in the tissue preparation can then be measured from this graph.

Fig. 3.2 illustrates the effect of (+)-carvone on intracellular cyclic AMP levels in the olfactory tissue. The results show that the cyclic AMP levels do not change in the presence of an odour at various concentrations. Liquid paraffin was used to dilute the neat odour solution, assumed to be 100 % (v/v), to its appropriate strengths. The tissue stimulation was attempted by suspending the intact turbinates

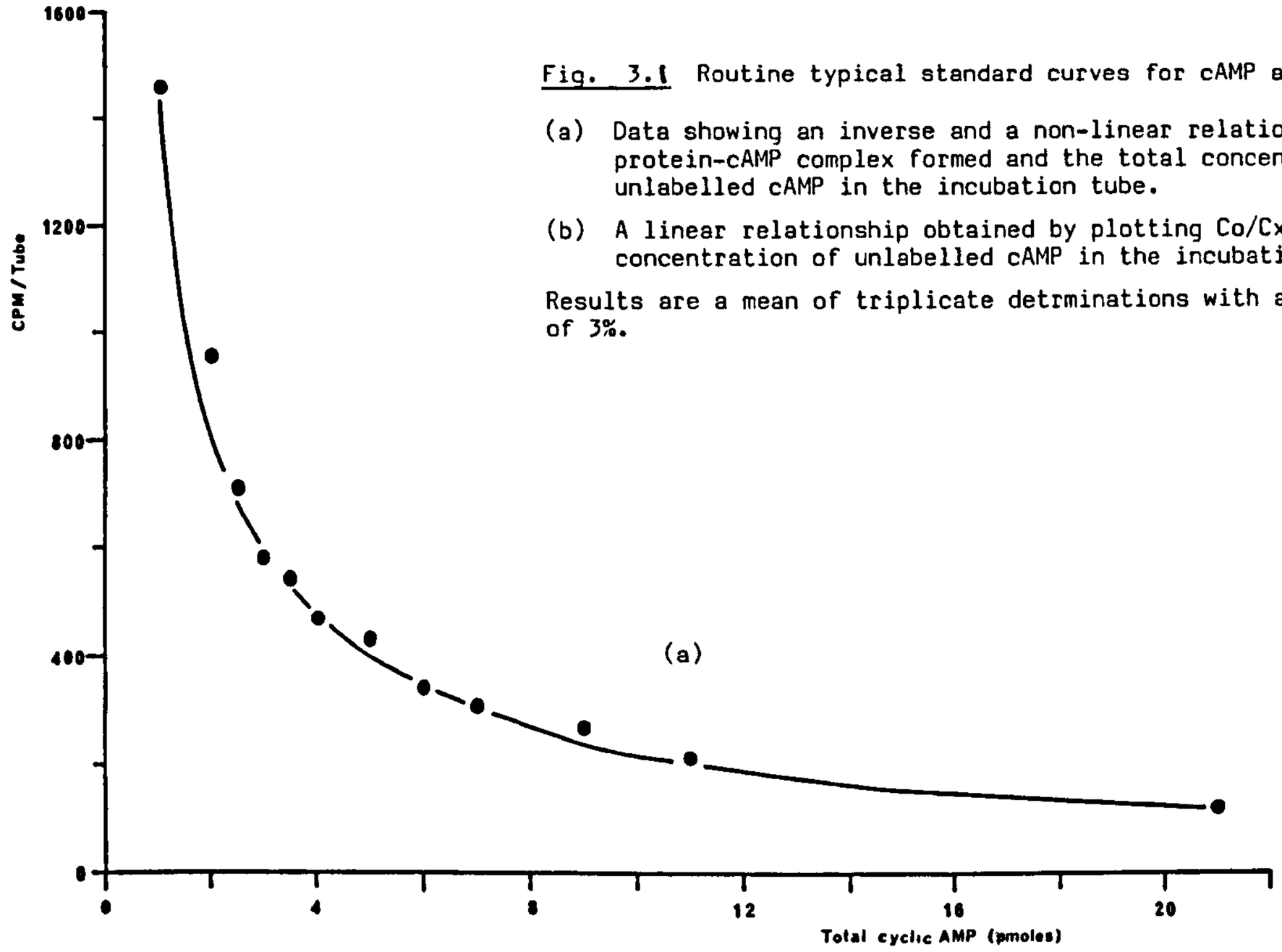
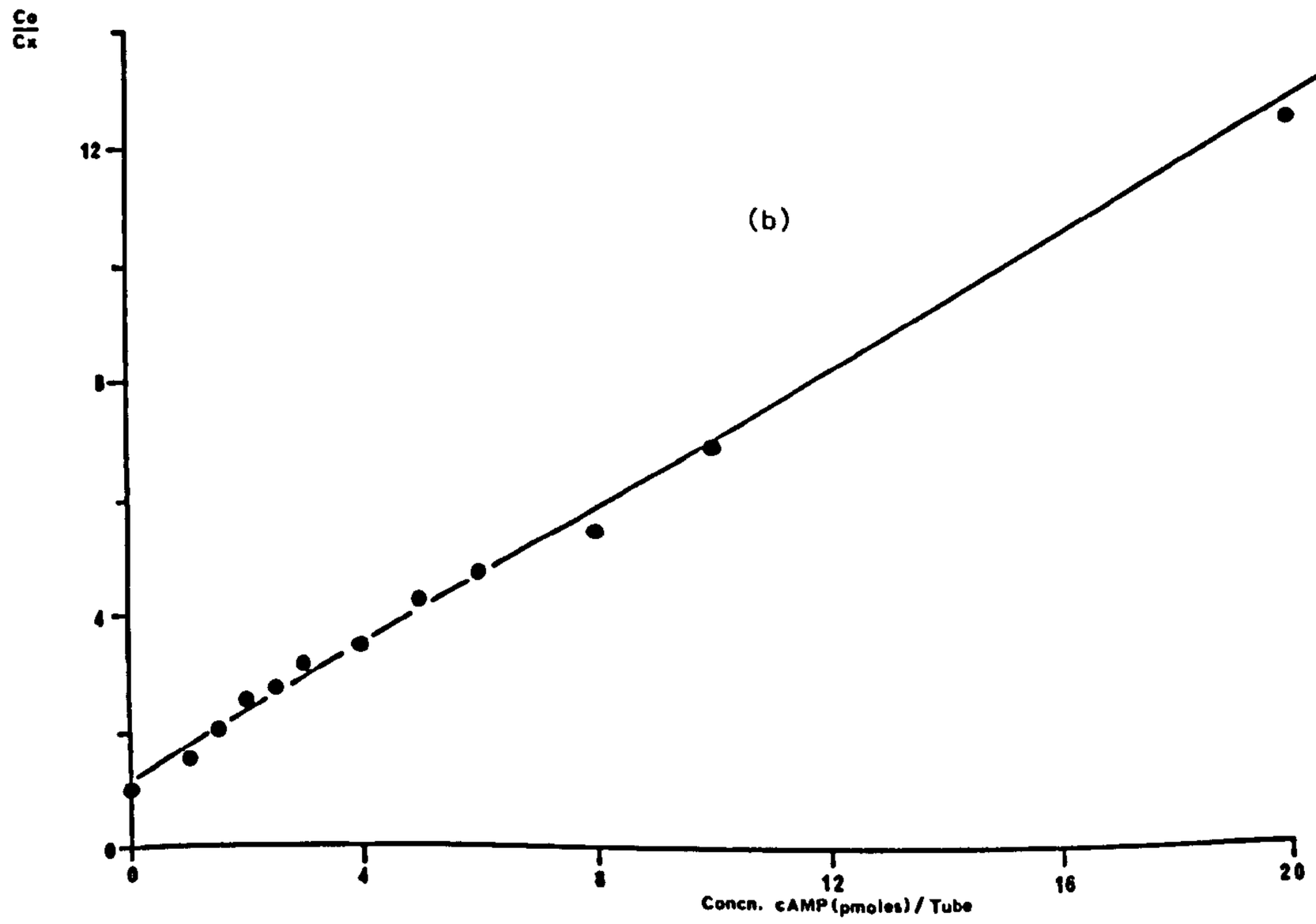


Fig. 3.1 Routine typical standard curves for cAMP assay.

(a) Data showing an inverse and a non-linear relationship between the protein-cAMP complex formed and the total concentration of unlabelled cAMP in the incubation tube.

(b) A linear relationship obtained by plotting Co/Cx against the total concentration of unlabelled cAMP in the incubation tube.

Results are a mean of triplicate detrminations with a standard deviation of 3%.



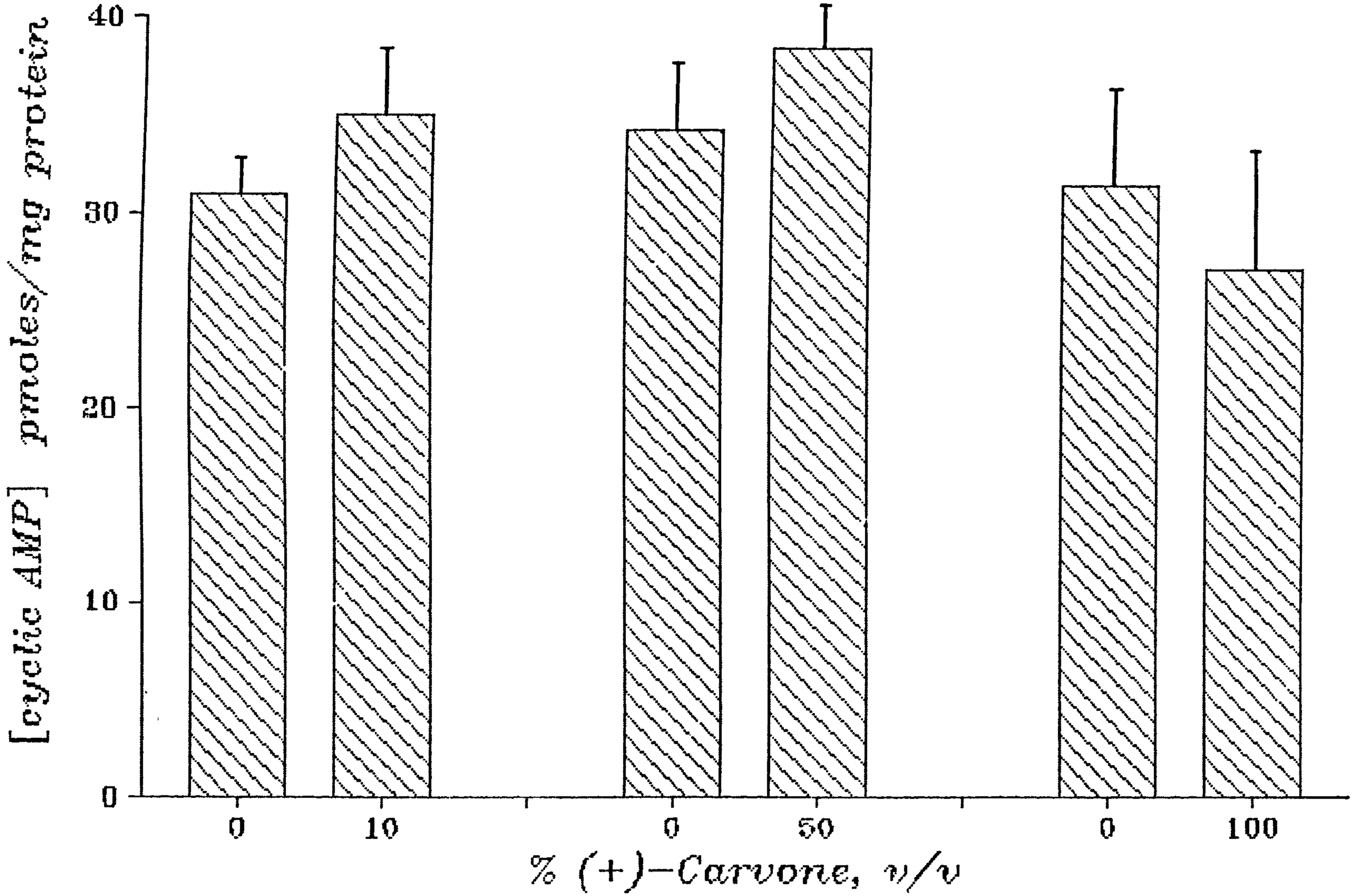
in the odorant vapour for 1 sec. It was observed that the protein concentrations of the tissue varied considerably (between 2.6 to 6 mg /turbinates from half rat head), even among turbinates from the same rat head. The tissue weights also varied considerably. Various modifiactions of the tissue preparation were thus investigated to minimize the material loss incurred.

It was decided that sample powdering may be unneccesary since the freeze-clamping may be sufficient to arrest the changes occurring in the turbinates. This was reasonable since the turbinates were thin enough for them to be frozen rapidly and for the trichloroacetic acid to quickly extract the cyclic AMP following sonication. However, as Fig. 3.3 illustrates, there are no significant differences in the cyclic AMP levels compared to those obtained with the powdered turbinates. Again the protein concentrations were variable and the tissue losses still occurred, suggesting that this process does not offer an improvement.

An extension of the above procedure was to avoid freeze-clamping and powdering completely, since these two steps are the most likely in which the material may be lost. Thus, the dissected turbinates were transferred directly into trichloro-acetic acid and immediately sonicated. It was found that the cyclic AMP levels in this preparation were very similar to those obtained in the earlier two preparations (see Fig. 3.4), and thus were unaffected in the presence of an odour. However,

Fig. 3.3 The effect of odour stimulation on intracellular cAMP levels of the rat olfactory tissue, using the freeze-clamping method.

Rat olfactory turbinates were stimulated for 1s by exposure to various concentrations of the odorant ((+)-carvone) vapour. Control tissues were held in air for the same length of time. Reactions were terminated by freeze-clamping the tissue and the cAMP extracted and assayed without powdering the tissue as detailed in methodology. Results represent means of triplicate determinations with standard deviation.



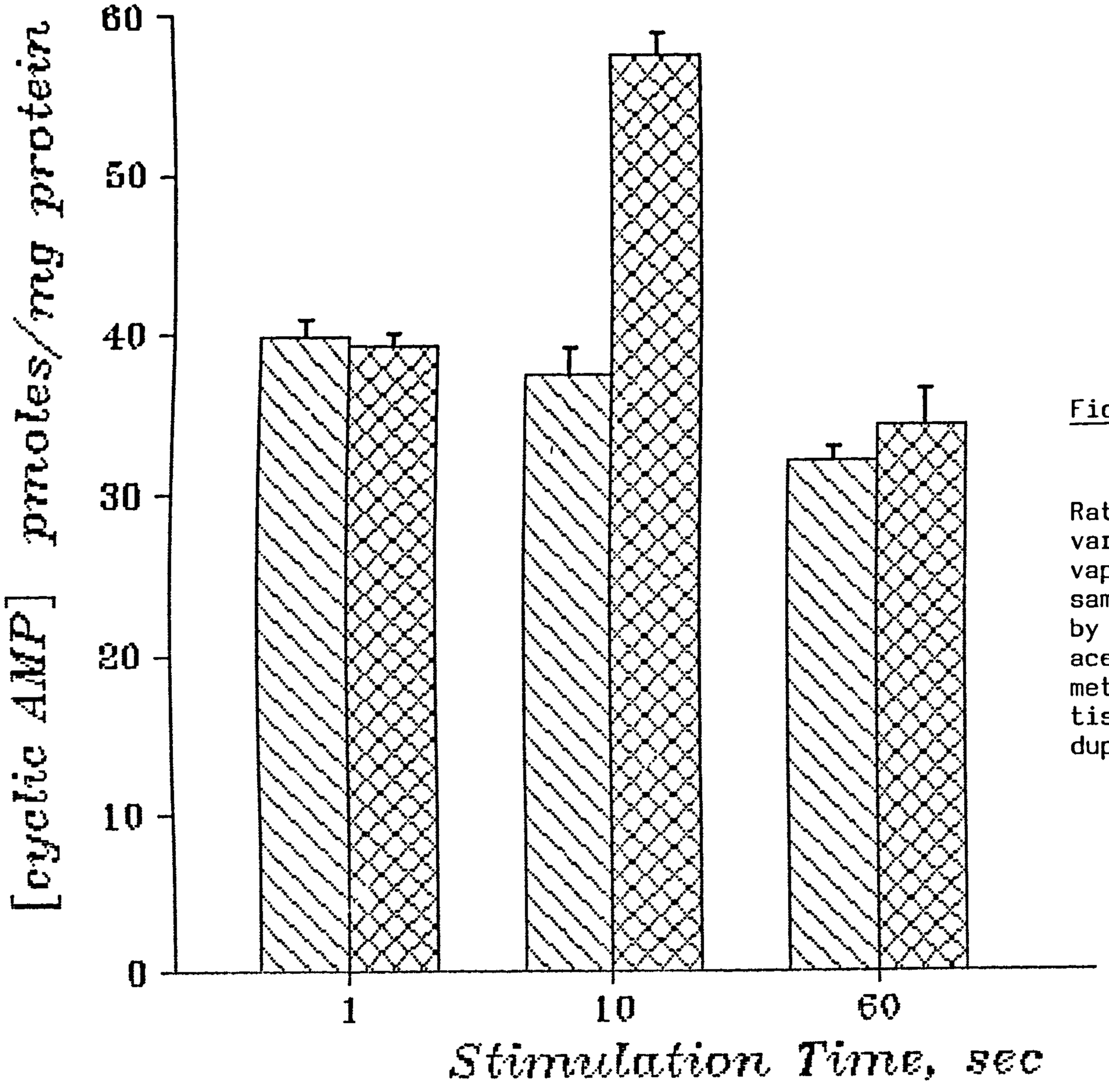


Fig. 3.4 The effect of different stimulation times by an odour on intracellular cAMP levels in the rat olfactory tissue.

Rat olfactory turbinates were stimulated for varying times by exposure to odorant ((+)-carvone) vapour. Control tissues were held in air for the same lengths of time. Reactions were terminated by extraction of cAMP with 10% v/v trichloroacetic acid and the cAMP assayed as detailed in methodology. No clamping or powdering of the tissue was attempted. Results are means of duplicate determinations with standard deviation.

in this procedure the material loss was considerably reduced. This figure also illustrates the effect of different odorant stimulation times on the intact unfrozen turbinates and shows that a 10 sec odorant exposure causes a 53 % stimulation of the cyclic AMP, whereas a 60 sec exposure produces a much smaller stimulation (see Fig. 3.4). It is not clear, however, whether these changes in cyclic AMP levels are due to the stimulation of adenylate cyclase or produced as a result of intrinsic variation in cyclic AMP levels in different tissues, since the control tissue was the remaining half of the rat head. Several attempts to reproduce the above variation in cyclic AMP levels following odorant stimulation proved fruitless.

The effect of IBMX and forskolin on the intracellular cyclic AMP levels was also investigated. The dissected turbinates were immersed in either a solution of IBMX (1mg/ml) or forskolin (100 uM) before being extracted for cyclic AMP. The control tissue was immersed in phosphate buffer (5mM, pH 7.5) with no other additions. The results indicate that the cyclic AMP levels are enhanced by both IBMX and forskolin (Fig. 3.5). IBMX is known to prevent the breakdown of cyclic AMP to 5-AMP (see Chapter 2) and forskolin directly activates adenylate cyclase. IBMX is seen to stimulate the cyclic AMP activity by 150 % and forskolin by 50 %.

The above experiment was then repeated in the presence of an odour (acetophenone, 1 mM solution in phosphate buffer).

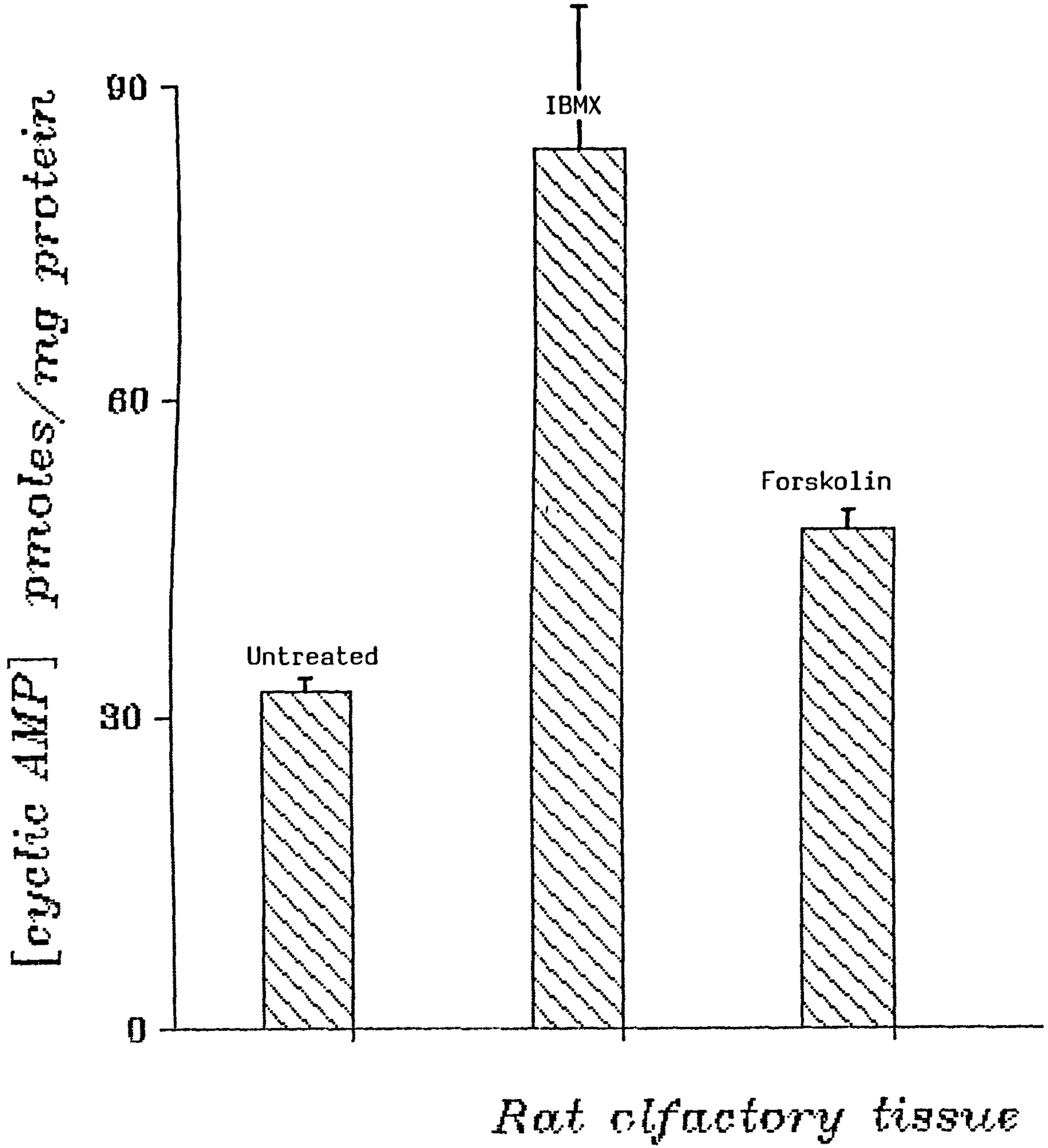


Fig. 3.5 The effect of IBMX and Forskolin on cAMP levels within the olfactory tissue.

Rat olfactory turbinates were stimulated by IBMX (1mg/ml) or forskolin (100uM) for a period of 2 min, by immersing tissues in phosphate buffer containing the appropriate compounds. Control tissues were immersed in phosphate buffer without other additions. Reactions were terminated by extraction of cAMP with trichloroacetic acid (10% v/v) and cAMP assayed as detailed in methodology. Results represent means of duplicate determinations with standard deviation.

The turbinates were stimulated by immersing in a solution of acetaphenone + IBMX for a period of 2 min. The control turbinates were soaked in a phosphate buffer + IBMX for the same period of time. The results indicate that the cyclic AMP level is not stimulated in the presence of this odorant (see Fig. 3.6). Higher levels of cyclic AMP are observed in the presence of IBMX, as expected.

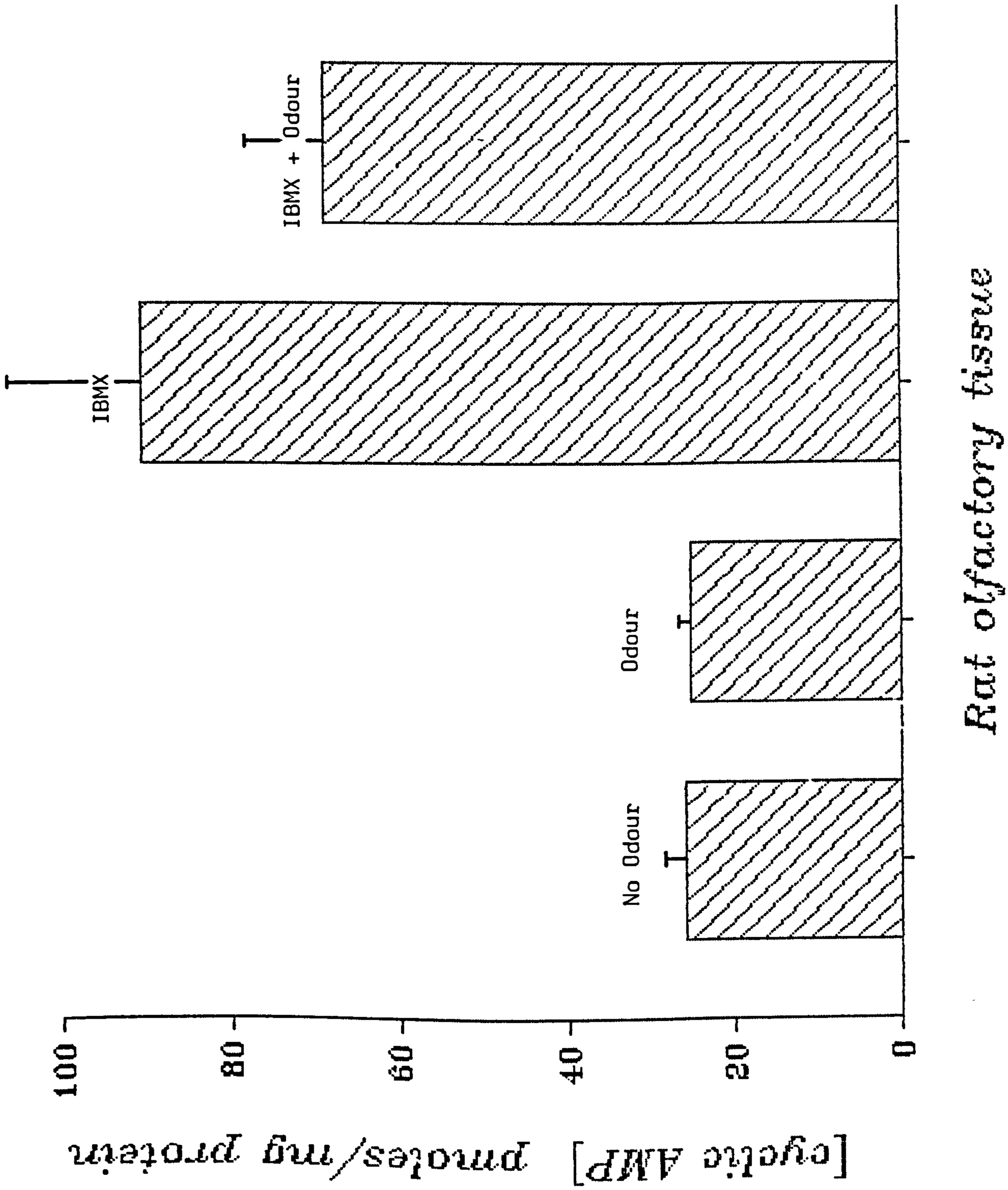
3.4 DISCUSSION

Attempts were made in this study to measure the changes in intra-cellular levels of cyclic AMP in the presence of odorants. However, this proved difficult owing to the problems associated with the tissue preparations.

The objective of freeze-clamping was to freeze the tissue rapidly enough to prevent artificial changes in cyclic nucleotide content, associated with excision or other disturbances of the normal milieu of the tissue. To prevent thawing of the tissue, the pestle and mortar were also cooled in liquid nitrogen. The time lapse from the moment the animal was killed to the moment the excised tissue was freeze-clamped is found to be a critical factor in preventing the variation in cyclic AMP due to the secondary effects. The dissected olfactory turbinates could only be freeze-clamped one at a time, giving rise to a dissimilar pair of turbinates. Thus, a direct comparison of these turbinates is not strictly valid.

Fig. 3.6 The effect of IBMX and the odour acetophenone on cAMP levels within the olfactory tissue of the rat.

Rat olfactory turbinates were incubated for 2 mins in phosphate buffer containing IBMX (1mg/ml) and the odour acetophenone (1uM). Control tissues were soaked in phosphate buffer containing IBMX (1mg/ml) only for the same length of time. Reactions were terminated by extraction of cAMP with trichloroacetic acid (10% v/v) and cAMP assayed as detailed in methodology. Results represent means of duplicate determinations with standard deviation.



A set of rat olfactory turbinates obtained from half a rat head is a very small tissue, usually weighing approximately 50 mg (wet weight), therefore providing only a small amount of powdered material. Thus transferring the powder from the mortar to the tube created some difficulties, i.e. the powder began to thaw very quickly and stick onto the base and sides of the mortar. This gave rise to heavy material losses, as indicated by the observed variation in the protein concentrations. This problem is not encountered with the brain tissue, which provided relatively large quantities of powder and the percentage losses observed were low.

An attempt to prevent the powder in the mortar from thawing was made by lining the mortar base with a piece of aluminium foil. Hence, once the turbinates were powdered it was possible to lift up the foil with forceps and transfer the material quickly and relatively completely into the tube containing the trichloroacetic acid. This technique proved to be a significant improvement in that the powder was transferred before it began to thaw and the material loss, although still incurred, was considerably reduced. It is important to note that the enzymes which may affect cyclic AMP concentrations must be inactivated before the sample begins to thaw.

The experiment, in which the freshly dissected tissue was transferred into trichloroacetic acid without either freezing or powdering, yielded consistent protein

concentrations. Furthermore, the material loss was reduced. The intracellular cyclic AMP levels in the whole-brain control tissue are found to be similar to those reported in the literature[10,11] (20.1 ± 2.3 pmole/mg protein), thus confirming the reliability of the cyclic AMP assay. The rat olfactory tissue appears to show much higher levels of the cyclic AMP.

However, the odour modulation experiments do not confirm the involvement of cyclic AMP in olfactory transduction. (+)-Carvone, an electrophysiologically proven olfactory odorant[12], does not produce any changes in the cyclic AMP levels, although in Chapter 2 (+)-Carvone is seen to enhance the adenylate cyclase activity. Similarly, the cyclic AMP levels also remain unchanged at different concentrations of this odour.

The lack of odorant stimulation observed could be due to the fact that any increase in the cyclic AMP levels may be "masked" by artificial increases of cyclic AMP that tend to occur upon decapitation and stress caused to the animal. Schmidt et al.[13] have shown that cyclic AMP concentrations in the brain rise rapidly following decapitation and also indicate that freeze-clamping does not provide a rapid inactivation of the formation/destruction of cyclic AMP. They suggest microwave radiation for rapid heat-inactivation. They conclude that nearly 100 % inactivation of the rat-brain adenylate cyclase and phosphodiesterase occurs after 20 sec of irradiation in a commercial oven. This method of killing the

animal appears to offer the advantages of rapid tissue fixation, easy dissection, and a minimum stress on death, the factors which can elevate the intracellular levels of cyclic AMP and therefore may swamp any changes that occur in the odour-modulated cyclic AMP levels.

Another reason for this undetected increase in the cyclic AMP levels may be its destruction by the phospho-diesterase enzyme as soon as it is produced. Indeed, work carried out in this laboratory (Keith Dickenson, personal communication) shows that the olfactory tissue contains a high level of phosphodiesterase activity. The use of a phosphodiesterase inhibitor, IBMX, elevates the cyclic AMP levels, however, these levels remain unchanged following odour modulation.

An alternative explanation for this unchanged levels of cyclic AMP following odour stimulation may lie in the fact that most of the cyclic AMP in the olfactory epithelium is bound to other molecules, leaving only a small concentration of free cyclic AMP. The biochemical techniques used measure the total amount of cyclic AMP and therefore may not detect the small changes that are physiologically important. Similarly, in a recent study on vision[14] where it was demonstrated that light stimulates a rapid breakdown of cyclic GMP, changes in levels of cyclic GMP were also not detectable.

It appears, therefore, that the use of a much more

sensitive technique is required to detect small changes in the intracellular cyclic AMP. In addition, various modifications of the experimental procedures discussed above are needed to reduce artificial fluctuations of cyclic AMP levels.

REFERENCES

1. Robinson, G.A., Butcher, R.W. and Sutherland, E.W. (1971) "Cyclic AMP", (Academic Press, New York-London, pp. 531.

2. Robinson, G.A., Exton, J.H., Park, C.R. and Sutherland, E.W. (1967) Fed. Proc., 26, 257.

3. Walsh, D.A., Perkins, J.P. and Krebs, E.G. (1968) J. Biol. Chem., 243, 3763.

4. Pace, U., Hanski, E. Soloman, Y. and Lancet, D. (1985) Nature (London), 316, 255-258.

5. Sklar, P.B., Anholt, R.R.H. and Snyder, S.H. (1986) J. Biol. Chem., 261, 15538-15543.

6. Mayer, S.F., Stull, J.T. and Wastilla, W.B. (1974) Meth. Enzymol., 38, 3-9.

7. Hartree, E.F. (1972) Anal. Biochem., 48, 422-427.

8. Gilman, A.G. (1970) Proc. Natl. Acad. Sci. U.S., 67, 305-312.

9. Walsh, D.A., Ashby, C.D., Gonzalez, C., Calkins, D., Fischer, E.H. and Krebs, E.G. (1971) J. Biol. Chem., 246, 1977.

10. Paul, M., Pauk, G., Ditzion, B.R. (1968) Pharmacol., 17, 2107.

11. Schmidt, M.J., Schmidt, D.E., Robinson, G.A. (1971) Science, 173, 1142-1143.

12. Shirley, S.G., Polak, E.H. and Dodd, G.H. (1983) Eur. J. Biochem., 132, 485-494.

13. Schmidt, M.J., Hopkins, J.T. Schmidt, D.E. and Robinson, G.A. (1972) Brain Res., 42, 465.

14. Vines, G. (1985) New Scientist, 1st August, 40-43.

CHAPTER 4

THE ROLE OF PHOSPHOLIPIDS IN OLFACTION

4.1 INTRODUCTION

4.1.1 PHOSPHOINOSITIDE METABOLISM

In recent years there has been a great deal of progress in the understanding of mechanisms by which cells respond to extracellular signals. These extracellular signals are transmitted across the cell membrane by a variety of mechanisms that utilise messenger molecules. The signalling system using cyclic AMP as the second messenger may be the best known. Until recently, very little has been known about the nature of the second messengers used by another major signalling pathway that utilises the inositol lipids as part of a transduction mechanism.

Stimulated inositide metabolism was descovered by Hokin & Hokin[1], who showed that the incorporation of ^{32}P into phopholipids in pancreas was stimulated by acetylcholine. Since then it has become apparent that a large number of different agonists can stimulate an increase in the metabolism of membrane phosphoinositides. These agonists include classical neurotransmitters such as acetylcholine, noradrenaline, histamine, 5-hydroxytryptamine, in addition to more complex molecules such as the peptides vasopressin,

substance P, angiotensin, pancreozymin, caerulein, thyrotropin-releasing hormone, platelet-derived growth factor and epidermal growth factor. In the well established areas of pharmacology, a change in phosphoinositide metabolism is always specific for one particular receptor class, such as the muscarinic cholinergic, the α_1-adrenergic, the H_1-histaminergic or the V_1-vasopressin receptor. A common feature of these receptors is that they are multifunctional in nature and are involved in general transduction mechanisms for the mobilisation of calcium, the activation of protein kinase C, the release of arachidonic acid and the activation of guanylate cyclase.

4.1.2 STRUCTURE OF THE PHOSPHOINOSITIDES

Phosphoinositides constitute 2 to 8 % of the lipid in cell membranes in eukaryotic cells and are essential for cell survival[2]. So far, the collective term phosphoinositide has been used to describe the three anionic phopholipids that contain myo-inositol in their head groups[3]. The most abundant form is phosphatidylinositol (PtdIns) that contains myo-inositol attached to phosphate through the hydroxyl on the 1-position (Fig. 4.1). The other two minority members are formed by sequential phosphorylation of hydroxyl groups on the 4- and 5-positions of PtdIns[4]. Membranes contain a PtdIns kinase (Fig. 4.1a) that specifically phosphorylates the hydroxyl on the 4-position to produce phosphatidylinositol 4-monophosphate (PtdIns4P). A PtdIns4P kinase (Fig. 4.1b) phosphorylates the

hydroxyl on the 5-position to form phosphatidylinositol 4,5-biphosphate (PtdIns4,5P_2). The conversion of PtdIns to these two polyphosphoinositides can be reversed by two phosphomonoesterases that specifically remove phosphate from the 5- and 4-positions (Fig. 4.1c & 4.1d).

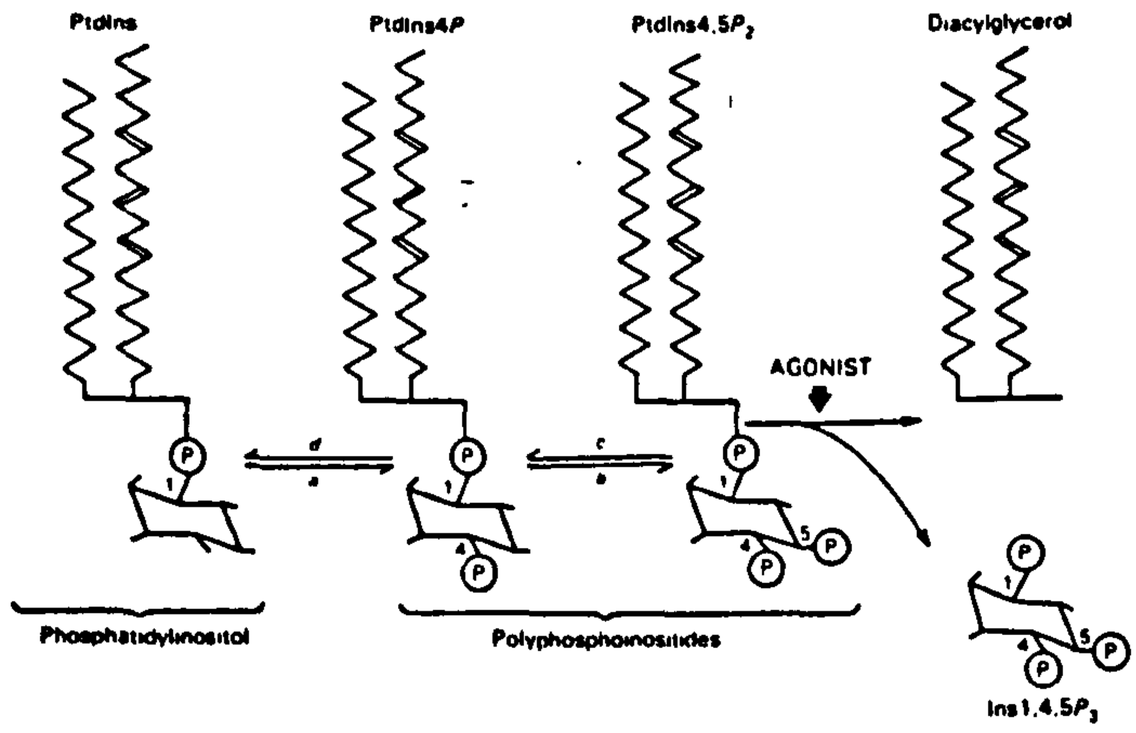


Fig. 4.1 Phosphorylation-dephosphorylation pathways of phosphoinositide metabolism.

These phosphoinositides are mainly confined to the inner leaflet of the plasma membrane. Agonists act by stimulating the hydrolysis of PtdIns4,5P_2 by a phosphodiesterase enzyme to give diacylglycerol and Ins1,4,5P_3.

The polyphosphoinositides represent 10 to 20 % of the inositol lipids in most cells. PtdIns4,5P_2 is usually less prevalent than PtdIns4P accounting for only 1 to 10 % that of the PtdIns content. Although the function of the phosphoinositides is only now being elucidated, their structures and routes of biosynthesis were elucidated around 1960. Kennedy and co-workers defined the pathway for PtdIns biosynthesis in microsomes[5], and the structures of the

polyphosphoinositides were established in a classical series of studies by Ballou and co-workers[5]. The methods that Ballou developed remain important tools in defining the structures of the newly discovered inositol phosphates described below.

4.1.3 THE MAIN PATHWAYS OF PHOSPHOINOSITIDE METABOLISM

The current view of the main pathways of phosphoinositide metabolism is shown in Figs. 4.2 and 4.3.

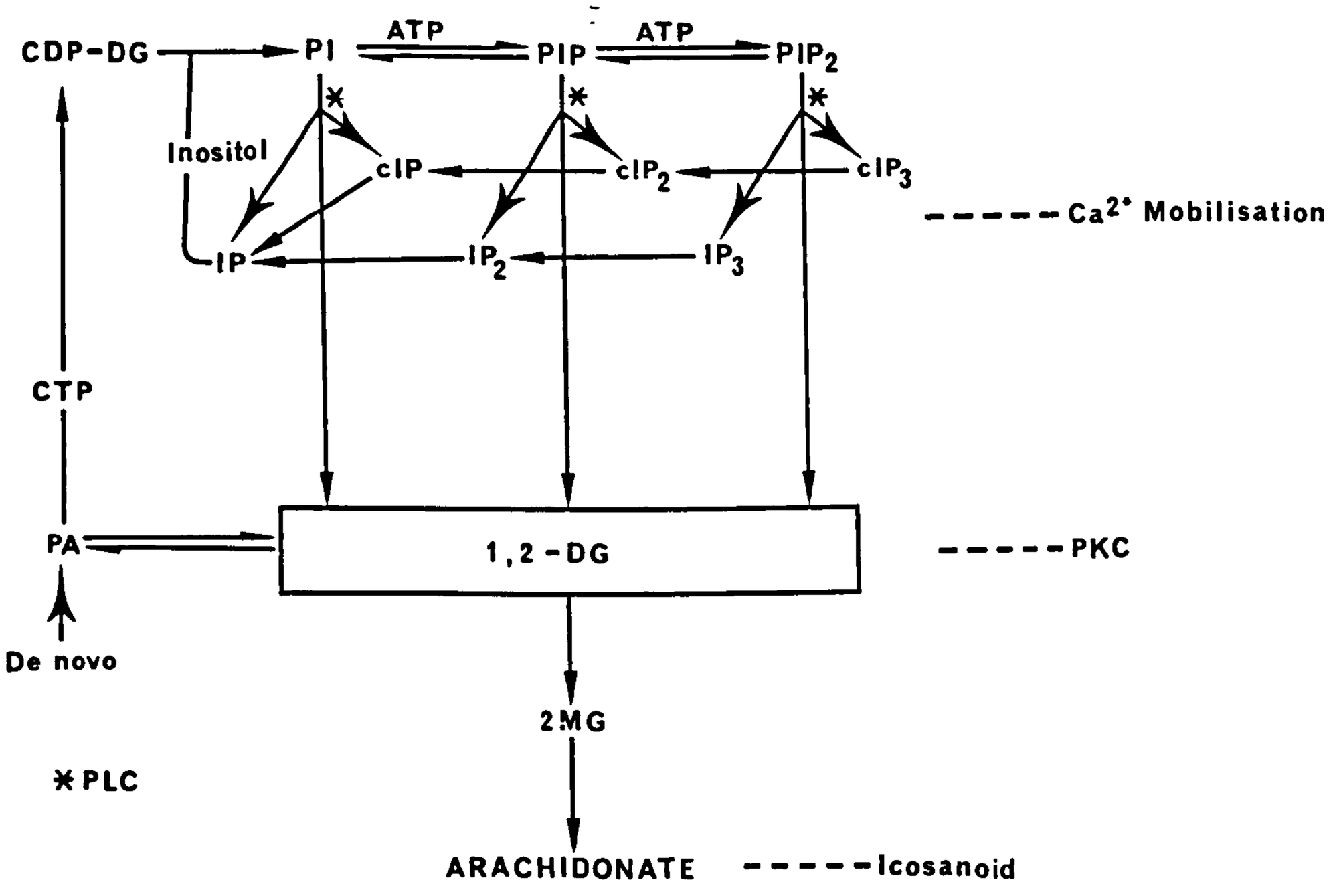


Fig. 4.2 Scheme for phosphoinositide breakdown and resynthesis. Messengers derived from phosphoinositides are shown on the right. PI, Phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP_2, Phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; cIP_3, inositol 1:2-cyclic 4,5-trisphosphate; IP_2, inositol 1,4-bisphosphate; cIP_2, inositol 1:2-cyclic 4-bisphosphate; IP, inositol 1-phosphate; cIP, inositol 1:2-cyclic phosphate; 1,2-DG, 1,2-diacylglycerol; PA, phosphatidic acid; CDP-DG, cytidine diphosphate diacylglycerol; 2MG, 2 monoacylglycerol, PKC, protein kinase C.

Phosphoinositides break down rapidly in response to occupancy of several types of receptors by specific agonists[7]. The three phosphoinositides are degraded by a phosphoinositide-specific phospholipase C (PLC) to form diacylglycerol (1,2-DG) and the various inositol phosphates. The inositol phosphates are rapidly degraded to inositol (I), which is utilised for resynthesis of phosphoinositides. Diacylglycerol is either hydrolysed by lipases to monoacylglycerol (2MG) and then to free arachidonate and glycerol or is phosphorylated by diacylglycerol kinase to form phosphatidic acid (PA), which is then used in the synthesis of phosphoinositides. At present, there are three messenger molecules known to be produced from phosphoinositides (described below), 1,2 diacylglycerol (1,2-DG), inositol 1,4,5 tris phosphate (Ins 1,4,5 P_3) and arachidonic acid (Fig. 4.2). Diacylglycerol acts as an essential cofactor for protein kinase C. Ins 1,4,5 P_3 functions as a messenger to mobilise Ca^{2+} from an intracellular site, probably in the endoplasmic reticulum, whilst arachidonic acid is oxygenated to form other mediators including prostaglandins, thromboxanes and leukotrienes.

Most early studies of phosphoinositide metabolism were confined to PtdIns itself. However. it is clear that the polyphosphoinositides are also degraded by PLC[8].

4.1.4 PRODUCTION OF 2ND MESSENGERS

4.1.4.i Diacylglcerol (DG)

Once DG and $Ins1,4,5P_3$ are produced by hydrolysing $PtdIns4,5P_2$ they are rapidly removed by various metabolic pathways some of which generate additional signals.

The neutral DG can be phosphorylated by a DG kinase to form phosphatidic acid (see Fig. 4.2) which has been implicated as an ionophore to gate calcium across the plasma membrane[9]. Alternatively, DG may be the substrate for a lipase which acts to release arachidonic acid which can then be converted to a variety of active metabolites including prostaglandins, leukotrienes and thromboxanes. The neutral DG which remains within the plane of the membrane, acts by stimulating protein kinase C[10] (see Fig. 4.3). Calcium and the phospholipid phosphatidyl serine (PS) are essential cofactors for this activation; these combine with DG and protein kinase C to form a quaternary complex within the membrane. During the formation of this active complex, protein kinase C moves from the cytosol and becomes bound to the membrane. Such cytosol-to-membrane translocation of protein kinase C have been reported in many different cells responding to a variety of external signals[11].

An important development in this field was the discovery that protein kinase C could be activated by the tumour-

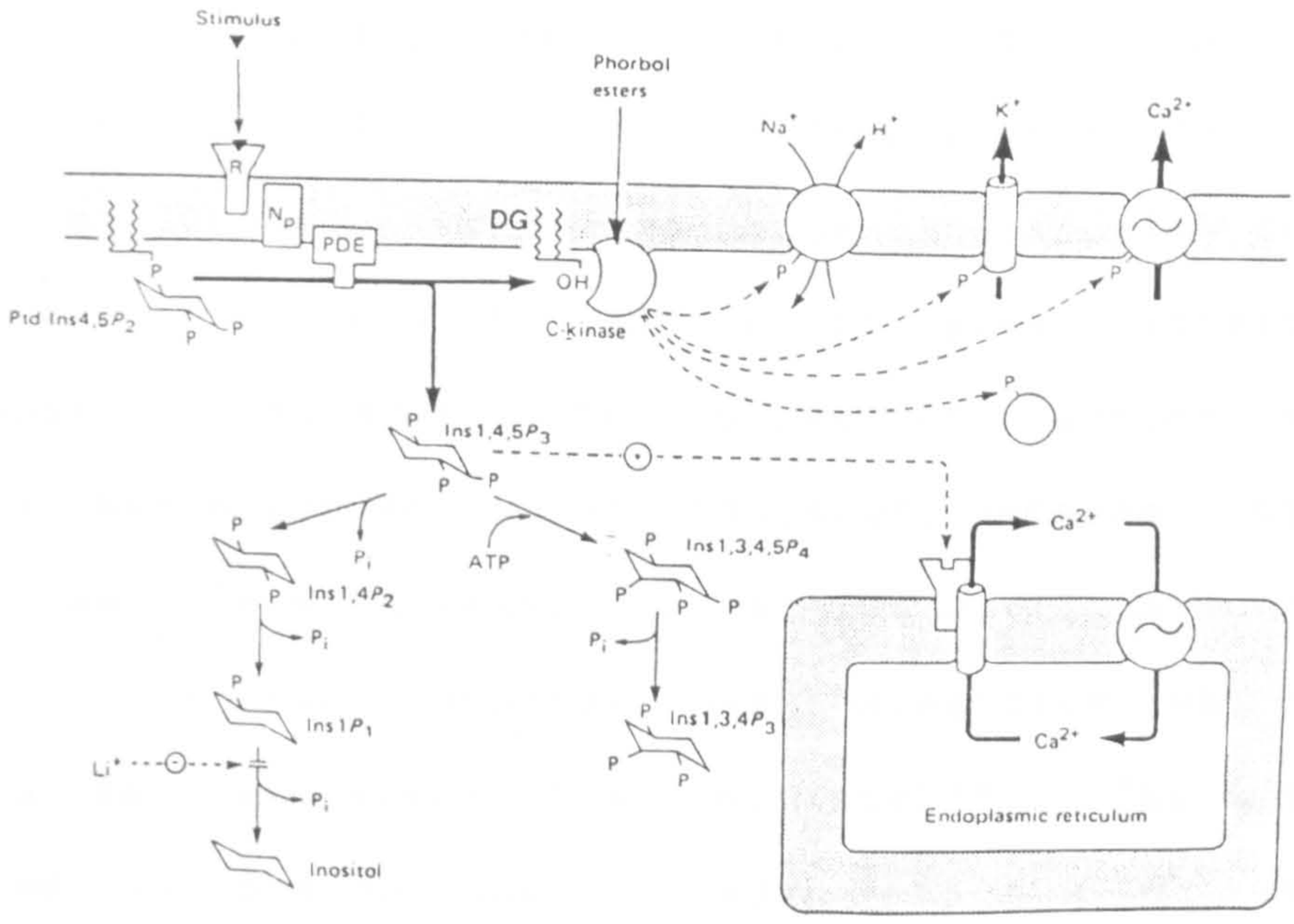


Fig. 4.3 The bifurcating signal pathway based on the agonist dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P_2). [From Berridge, M.J. (1986) Biol. Chem. 367, p448.

External stimuli acting on surface receptors (R) are coupled through a GTP-binding protein (N_p) to the phosphodiesterase (PDE) which cleaves PtdIns4,5P_2 to give diacylglycerol (DG) and inositol trisphosphate (Ins1,4,5P_3). These two products have separate functions within the cell with DG activating a protein kinase (C-kinase) while Ins1,4,5P_3 acts to release calcium from the endoplasmic reticulum.

promoting phorbol esters[12]. Phorbol esters can thus be used to mimic the action of DG and provide a pharmacological tool whereby protein kinase C can be activated independently of the Ins1,4,5P_3/Ca^{2+} pathway.

4.1.4.ii Inositol 1,4,5-Tris Phosphate ($Ins1,4,5P_3$)

The $Ins1,4,5P_3$ released to the cytosol can be metabolised via two separate pathways (see Fig. 4.3). A series of phosphatases result in the stepwise dephosphorylation of $Ins1,4,5P_3$ to free inositol[13]. The first enzyme, an inositol trisphosphatase specifically removes the phosphate from the 5-position to give inositol 1,4-bisphosphate ($Ins1,4P_2$). This enzymatic reaction terminates the 2nd messenger action of $Ins1,4,5P_3$ because $Ins1,4P_2$ is totally inactive with regard to calcium mobilisation[14]. There appear to be two inositol bisphosphatases which act on $Ins1,4P_2$ to give either InsIP or Ins4P[13]. The sequence is completed by inositol monophosphoesterases which remove the phosphates from these two monophosphates to give free inositol. The enzyme which dephosphorylates InsIP is very sensitive to lithium[15].

At present, the only inositol phosphate for which a 2nd messenger function has been identified is either $Ins1,4,5P_3$[14] or its cyclic derivative[16]. Recently it has been shown that when $PtdIns4,5P_2$ is cleaved in an <u>in vitro</u> system, both $Ins1,4,5P_3$ and inositol 1,2(cyclic)-4,5-trisphosphate (cyclic $Ins1,4,5P_3$) are formed[17]. The latter must also be considered as a 2nd messenger because although it is less effective than $Ins1,4,5P_3$ in mobilising calcium it is more effective in stimulating Limulus photoreceptors[18].

An alternative pathway for the metabolism of Ins1,4,5 P_3 is thought to occur as follows:

$$\text{Ins 1,4,5 P3} \xrightarrow[\text{kinase}]{\text{ATP}} \text{Ins 1,3,4,5 } P_4 \xrightarrow{\text{5-PME}} \text{Ins 1,3,4 } P_3$$

An inositol trisphosphate 3-kinase adds the phosphate to the 3- position to give inositol 1,3,4,5 tetrakisphosphate (Ins 1,3,4,5 P_4). The latter has recently been identified in brain corticol slices[19] and in a number of other tissues[19]. In addition to Ins 1,3,4,5 P_4 many of these tissues also have Ins P_5 and Ins P_6 (phytic acid)[19]. There is evidence that Ins 1,3,4,5 P_4 is dephosphorylated by an inositol trisphosphate 5-phosphomonoesterase (5-PME) to form the Ins 1,3,4 P_3, which has been identified in a number of tissues[20]. However, further metabolism of Ins 1,3,4 P_3 has not yet been determined. Whether Ins 1,3,4,5 P_4 or Ins 1,3,4 P_3 are themselves distinct messenger molecules is uncertain.

4.1.4.iii Icosanoid Messengers

Icosanoid mediators (oxygenated derivatives of arachidonic acid and related polyunsaturated fatty acids, including prostaglandins, thromboxanes and leukotrienes) are produced by the release of arachidonate from phospholipids[21]. This release of arachidonate is associated with accelerated phosphoinositide turnover. Arachidonate is partly derived from phosphatidyl inositol itself by a series of reactions involving PLC and diacylglycerol and monoacylglycerol

lipases[22].

PI-----> 1,2-DG----------->2MG----------->arachidonate + glycerol
PLC 1,2-DG lipase 2 MG lipase

The fraction of arachidonate released from PI versus other phospholipids varies widely, depending on the cell type and the stimulus[21].

Arachidonate is also liberated by phospholipase A2[23]. In most cell types it is difficult to estimate accurately the source of arachidonate because a small fraction of the total cell arachidonate in any particular phospholipid is liberated.

4.1.5 PHOSPHOLIPASE C (PLC)

Phosphoinositide-specific phospholipase C is the enzyme that generates phosphoinositide derived messenger molecules. It cleaves phosphoinositides to yield 1,2-diacylglycerol and inositol phosphates. The enzyme is present in most cell types and most of the activity is cytosolic, although its substrates are in a membrane bilayer.

Two distinct soluble phospholipase C enzymes have been identified in ram seminal vesicles[24], designated PLC-I and PLC-II. Both appear to have similar activities but the tissue distribution of the enzymes differs; PLC-I is the predominant form inliver, and PLC-II is the major form in platelets and

brain. Multiple forms of soluble PLC also occur in partially purified preparations from other tissues[25]. The relationship between these multiple forms of enzyme and those described above is unclear. The PLC enzymes are specific for PtdIns and the polyphosphoinositides. They do not hydrolyse other phospholipids with the exception of phosphatidylglycerol, which is utilised 0.001 times as well as PtdIns[24]. The fact that the enzymes in <u>in-vitro</u> assays require Ca^{2+} to hydrolyse PtdIns readily distinguishes them from lysosomal PLC, which is not specific for PtdIns nor inhibited by Ca^{2+} chelation. Both PLC-I and PLC-II readily hydrolyse all three phosphoinositides[25].

4.1.6 THE REGULATION OF PHOSPHOLIPASE C ACTIVITY

4.1.6.i Membrane Lipids

Phospholipase C activity can be regulated by membrane lipids. These PLC enzymes are inactive unless bound to a lipid bilayer containing an appropriate substrate[27]. High contents of phosphatidylcholine (PC) in membranes markedly inhibit PLC by shielding the substrates from the enzyme and therefore preventing the enzyme from binding to PC-containing PtdIns vesicles[28]. PC inhibits polyphosphoinositide hydrolysis less than PtdIns hydrolysis. Phosphatidylserine, diacylglycerol, or free fatty acids can reverse the inhibition of PLC activity by PC; the role of these substances in the control of PLC <u>in-vivo</u>, however, is

unknown. It is interesting to note that diacylglycerol, phosphatidylserine, and free fatty acid also stimulate protein kinase C activity[10]. This latter enzyme is also cytosolic in location and is active only when bound to membranes.

4.1.6.ii Calcium

The Ca^{2+} ion concentration determines both the rate of hydrolysis of PtdIns and the preferred substrate in <u>in-vitro</u> assays. The ratio of PtdIns to PtdIns4,5P_2 in unilamellar vesicles made from total platelet lipids is approximately 14. At 100 uM Ca^{2+} ion concentration PLC from platelets cleaves PtdIns 20 times faster than PtdIns 4,5 P_2. In contrast, at 0.1 uM Ca^{2+}, the two substrates are cleaved equally well even though the concentration of PtdIns is 14 times higher. Although Ca^{2+} stimulates a severalfold increase in polyphosphoinositide hydrolysis in in-vitro reactions, the reaction proceeds even in the presence of EGTA[25]. The finding that PtdIns 4,5 P_2 hydrolysis occurs at low Ca^{2+} is consistent with the hypothesis that this reaction triggers Ca^{2+} mobilization in the cell. PtdIns breakdown requires Ca^{2+} in <u>in-vitro</u> assays and presumably the Ca^{2+} flux stimulated by Ins1,4,5P_3 initiates PtdIns hydrolysis. However, the role of Ca^{2+} in controlling PtdIns hydrolysis in intact cells is uncertain.

Increased intracellular levels of cyclic AMP in platelets block phosphoinositide breakdown, arachidonate

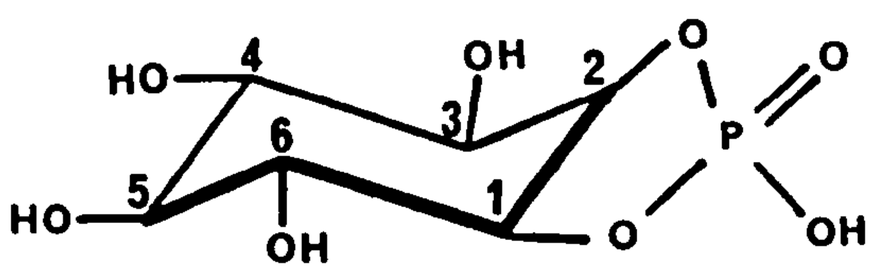
release, and secretion of granule contents in response to agonists. The mechanism by which cyclic AMP blocks phosphoinositide turnover is unknown. An inhibition of PLC by cyclic AMP has been proposed[28], although cyclic AMP has no direct effect on purified PLC as assayed in vitro. Alternatively, it has been suggested that cyclic AMP may act by preventing the rise in intracellular Ca^{2+} necessary for a response to stimulation[28].

4.1.6.iii G-Proteins

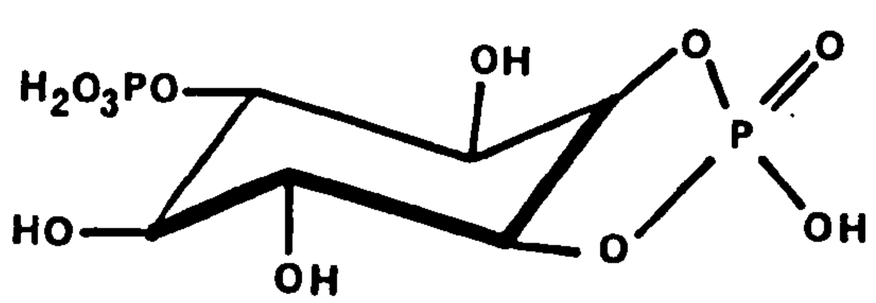
A role for guanine nucleotide binding proteins (G proteins) in the activation of PLC was initially suggested by studies showing that guanine nucleotides reduce the Ca^{2+} requirement for secretion in permeabilized mast cells and platelets[30]. Litosch et al.[31] demonstrated that serotonin promoted the breakdown of phosphoinositides by PLC in membranes from blowfly salivary glands and that guanine nucleotides potentiated this response. The ability of nonhydrolysable analogues of guanosine triphosphate (GTP) to stimulate breakdown of endogenous phosphoinositides has now been demonstrated in many systems, including human neutrophils[32], hepatocyte membranes[33], cerebral cortex membranes[34], and GH_3 pituitary cells[35]. These studies have led to the concept that the control of PLC may occur in a manner analogous to that of adenylate cyclase (see chapter 2).

4.1.7 INOSITOL CYCLIC PHOSPHATES

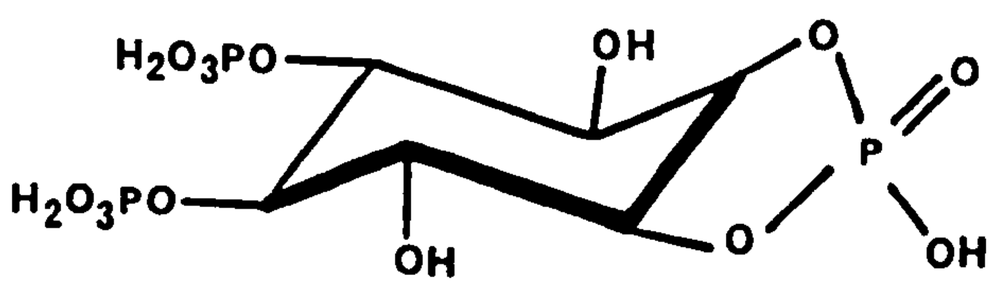
The inositol phosphate product of PLC hydrolysis of PtdIns is a mixture of Ins 1P and an inositol cyclic phosphate ester (cyclic Ins1P). All three phosphoinositides are utilised as subrates by both PLC-I and PLC-II therefore suggesting that these enzymes also form cyclic esters from PtdIns4P and PtdIns4,5P_2. Wilson et al.[36] have demonstrated that PLC produces cyclic products from all three phosphoinositide substrates; the structures of these are given below.



1,2 (cyclic)IP_1



1,2 (cyclic)IP_2



1,2(cyclic)IP_3

The isolation of the inositol cyclic phosphate products by high-performance liquid chromatography (HPLC)[37] reveals that 60 to 70% cyclic product is formed from PtdIns, 40 to 50% from PtdIns4P, and 30 to 40% from PtdIns4,5P_2. However, the basis for the difference in proportion of cyclic and noncyclic

inositol phosphate products with the various substrates remain unexplained. Both PLC-I and PLC-II produce similar amounts of cyclic and noncyclic products.

The physiological effects of the inositol cyclic phosphates have been examined in several systems. A number of studies have demonstrated that addition of $Ins1,4,5P_3$ to permeabilized cells results in mobilization of Ca^{2+} from intracellular stores. Permeabilised platelets release calcium in response to $Ins1,4,5P_3$ at micromolar concentrations[36]. Inositol 1,2(cyclic)4,5-trishosphate (cyclic $Ins1,4,5P_3$) is as potent as $Ins1,4,5P_3$ in platelets and in 3T3 cells in this assay, while inositol 1,2(cyclic)4-bisphosphate (cyclic $Ins1,4P_2$) is inactive[37]. The cyclic trisphosphate was approximately five times more potent than its noncyclic counterpart in stimulating limulus photoreceptors[18].

4.1.8 PHOSPHOINOSITIDE TURNOVER AND CELL GROWTH

Several growth factors, including platelet derived growth factor and epidermal growth factor, stimulate phosphoinositide turnover[38]. Furthermore, the tumour-promoting phorbol esters stimulate protein kinase C directly. It is thus thought possible that any changes in the phosphoinositide signal pathway could affect the control of cell growth. It is possible that derangements of the phosphoinositide messenger system may be important in the development of uncontrolled growth in transformed cells.

4.1.9 LIPIDS IN OLFACTION

One of the rewarding recent developments in olfactory biochemistry has been the elucidation of an olfactory transduction mechanism. Accumulating data suggest that cyclic AMP serves as a second messenger in odorant activation of the chemosensory neurons[40,41] (see chapter 2). However it has been shown that not all odorants activate the olfactory adenylate cyclase[41], therefore suggesting the possibility that different transduction mechanisms may be involved in olfaction. Recently, Huque and Bruch[42] reported that L-alanine, an amino-acid odorant, activates phosphatidylinositol 4-5-bisphosphate phosphodiesterase (PLC) in fish olfactory cilia. Furthermore, Anholt et al.[43] report the presence of protein kinase C in the frog olfactory epithelium, but its role in olfaction has not yet been established.

Membrane lipids are known to participate in cellular transductive events. While it is assumed that the plasma portion of the membrane plays a primary role in the recognition of most chemical stimuli[44], it is possible that the lipids of the plasma membrane are partially responsible for the recognition steps in certain olfactory responses. There is however a lack of basic knowledge of the type , concentration and metabolism of lipids within the olfactory epithelium.

In 1972, Koyama and Kurihara[45] examined, in humans, the

interaction of various odorants with the lipid monolayer.The findings suggested that an interaction between odorants and lipids in olfactory receptor membranes is important for olfactory reception. Cherry et al.[46] in 1970 and Fesenko et al.[47] in 1977 examined the effects of odorants on planar lipid membranes but did not demonstrate that odorants cause changes in the membrane potential of the lipid membranes. More recently, Nomura and Kurihara[48] have demonstrated that lipid membranes are depolarised by various odorants and suggest that odorant reception in the olfactory system is induced by adsorption of odorants onto the hydrophobic region of the lipid layer of the receptor membranes. In further studies, they showed that changes in lipid composition of liposomes greatly affected the magnitude and the direction of membrane potential changes in response to odorants. Their results support the mechanism of a nonreceptor-mediated odour discrimination for general odorants.

The following experiments aim to investigate the role of lipids in olfaction. The study initially required investigations on the type and concentration of lipid within the olfactory epithelium and is followed by studies on lipid metabolism.

4.2 METHODOLOGY

4.2.1 ANIMALS

Male Wistar rats of about 300g body weight were used; the animals were allowed food and water prior to experiments.

4.2.2 MATERIALS

Standard phospholipids including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidic acid (PA),phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), phosphatidylinositol 4,5-bisphosphate (PIP_2) and sphingomyelin (SM) were purchased from Sigma Chem. Co. Ltd. All solvents used for lipid extraction and chromatography were of analytical grade and purchased from Fisons. All solvents used for GC/MS were of GLC grade and were supplied by Fisons. Meth-Prep-II™ esterification reagent was supplied by Field Analytical Co. Ltd. BF_3- MeOH complex was purchased from BDH. Scintillation cocktail-SAFE was obtained from Fisons. Inorganic ^{32}P orthophosphoric acid was purchased from Amersham International. All other chemicals were obtained from Sigma Chemical Co. Ltd. and were of the highest commercially available quality. Silica gel 60 thin layer chromatography glass plates were obtained from E.Merck Co., Darmstad,Germany. Autoradiography X-ray developer, X-ray liquid fixer and photo-flo 200 were obtained from Eastman Kodak Co. Phase-Separator filter paper(9cm) was purchased from

Whatman. "Crimp" vials and teflon seals were supplied by Hewlett Packard.

4.2.3 PREPARATION OF NASAL EPITHELIUM

The animals were killed by cervical dislocation and decapitation. After sagittal sectioning of the head the ethmoturbinates were removed and soaked in cold phosphate buffer (composition 150mM NaCl, 5.63mM KCl and 2mM $NaH^{32}PO4$), pH adjusted to 7.0 with 1000mM NaOH. The solution was changed twice with minimal agitation to remove superficial blood and debris, before proceeding with the extraction of the phospholipids.

4.2.4 INCORPORATION OF ^{32}P INTO PHOSPHOLIPID

The ethmoturbinates were removed from a freshly killed rat, soaked in 5ml of ice-cold EDTA-Ringer solution (Composition NaCl 155mM, KCl 5.6mM, EDTA 1mM and Tris-HCl 10mM), pH adjusted to 7.8. The tissue was washed twice with 5ml of ice-cold Ringer (i.e. EDTA-Ringer solution with the EDTA replaced by 2.1 mM $CaCl_2$) and then left standing in a small amount of buffer on ice until the incubation was initiated. Typical wet tissue weights varied from 50-100 mg. The incubation was performed by replacing the media surrounding the washed tissue with 1ml of Ringer containing a known amount of ^{32}P orthophosphate (10-100 uCi). The head space above the tissue was replaced with oxygen containing 5%

carbon dioxide and the tissue preparations were incubated in a shaking water-bath (30°C, 120 strokes/min) for up to a period of 5 hours. The head space of oxygen/carbon dioxide above the tissue was refreshed every 30 min. The incubation was terminated by addition of 5ml of ice-cold Ringer and the tissue was washed twice more with fresh Ringer solution to remove as much of the unincorporated activity as possible prior to phospholipid extraction.

4.2.5 EXTRACTION OF PHOSPHOLIPIDS

4.2.5.i Acid Extraction

The method used was essentially that of Simpson and Sargent[48], but with a few modifications (described below). After removal of the aqueous wash-phase, 5ml of chloroform-methanol (2:1, v/v) solution and 0.4ml of 1M HCl was added to the tissue and the mixture immediately sonicated. Sonication was for 3x5s on ice, at the medium power setting of the M.S.E. 100W disintegrator using an exponentially tapered probe of 3mm tip diameter (In experiments where tissues were labelled with ^{32}P orthophosphate, as indicated in the results section, sonication was for 2x30s at the medium power setting, using a straight probe of 8mm diameter). The mixture was centrifuged at 1000xg for five minutes and the lower organic phase was removed and washed with 1ml of 0.3M HCl. After recentrifuging the lower phase was filtered through phase separating paper, dried under nitrogen and stored overnight under a vacuum.

4.2.5.ii Neutral Extraction

This method was a modification of the procedure adopted by Folch et al.[50]. After removal of the aqueous wash-phase, chloroform-methanol (5ml, 2:1 v/v) was added to the tissue and the mixture was sonicated as described above. KCl (1ml, 0.88% w/v) was then added to the sonicate and the mixture was vortexed for 30 s. After allowing the mixture to stand for 10 mins. it was centrifuged at 1000xg for 5 mins. and the lower phase filtered through phase-separating paper. The filtrates were dried under nitrogen atmosphere and stored overnight under a vacuum.

4.2.6 ISOLATION OF PHOSPHOLIPIDS

For the isolation of PA, PC, PE, PI, PS and SM, the lipid extract was chromatograhed on 20x20 cm thin layers of silica gel 60, 0.25 mm thick in two dimensions, using chloroform-methanol-water-ammonia sp.gr. 0.88, (130:70:8:0.5 by vol.) in the first dimension and chloroform-acetone-methanol-glacial acetic acid-H20 (10:4:2:2: 1 by vol.) in the second dimension[51].

For the separation of PA, PI, PIP and PIP_2, chromatography was carried out on the above 20x20 cm TLC plates, as well as on 10x10 cm plates. HPTLC plates coated with silica gel G 60 in chloroform-methanol-3.3M ammonia (43:38:12 by vol.) in the first dimension and chloroform-acetone-

methanol-glacial acetic acid-water (10:4:2:2:1 by vol) in the second dimension[52]. Prior to the experiment, the plates were impregnated with potassium oxalate by spraying each plate with a solution of methanol-water (2:3 v/v) containing 1% (w/v) potassium oxalate and reactivating at 110°C for 15 minutes[53]. For both separation procedures, the plates were loaded with 100-250 ug of lipid extract and the spots dried under a stream of nitrogen. After the first dimension was complete, the plates were allowed to dry in air for 30 minutes before commencing the second dimension. Major phospholipid spots were visualised by iodine staining. Minor phospholipids, including the phosphoinositides and phosphatidic acid were located by autoradiography.

4.2.7 AUTORADIOGRAPHY

After 2D chromatography was complete, the plates were allowed to dry in air for 30 mins. Each TLC plate was then marked with a ^{14}C labelled stamp for later identification. The plate was covered with Kodak x-omat AR 5 x-ray film, which was "sandwiched" by placing a plane glass plate on top of the film. The film was cut to size and the plates were held in position by bands. The plates were left undisturbed in a 'black box', (each set of plates was separated by lead sheets in order to prevent "inter-plate" fogging) for a period of 24 hours, unless otherwise stated.

The film was then developed in Kodak Lx-24 x-ray

developer and fixed in Kodak Fx-40 x-ray liquid fixer. After washing and drying, phospholipid spots were identified and marked on to the TLC plates by superimposition.

4.2.8 MEASUREMENT OF PHOSPHOLIPID PHOSPHORUS

The percentage composition of olfactory tissue phospholipid was determined by measuring phospholipid phosphorus in each sample, using the methods of Bartlett and Pollet et al.[54,55], with modifications (described below).

After detection, either by iodine or by autoradiography, individual phospholipid spots were scraped from the thin layer plates into appropriate tubes and eluted with 3x10 ml of chloroform-methanol-water (5:5:1 v/v), containing 0.01% (w/v) BHT. The solvent phase was removed under nitrogen. The dried-down phospholipids were then charred in an oven at 300°C for a period of 21 hours. After cooling, phospholipids were digested with 0.4 ml of 70% perchloric acid at 100°C for 20 minutes. 2.4 ml of ammonium molybdate reagent and 2.4 ml of reducing reagent were added to the mixture, and the colour developed by mixing thoroughly and heating at 100°C for a further 10 min. The ammonium molybdate reagent is prepared by dissolving the compound(4.4g) in water(500 ml), adding concentrated sulphuric acid (14ml), and making up to 1 litre. The reducing reagent consists of sodium bisulphite (2.5g), sodium sulphite (0.5g) and 1-amino-2-napthol-4-sulphonic acid (0,042g), which are dissolved in water (250 ml) and allowed to

stand in the dark for several hours. The solution was filtered into a brown bottle and when refrigerated was found to be stable for one month. The tubes were cooled and centrifuged at 1000 rpm for 10 mins. to separate out any silica particles carried across. The inorganic phosphate released was measured spectrophotometrically at 830 nm. against a reagent blank i.e the above mixture without the phospholipid sample. In each determination, reagent blanks and a series of standard inorganic phosphate solution of NaH_2PO_4 (0-10ug Pi), were treated similarly.

4.2.9 MEASUREMENT OF ^{32}P LEVELS INCORPORATED INTO PHOSPHOLIPID

After detection by autoradiography, each phospholipid spot was scraped from the TLC plate directly into a scintillation vial. 10 ml of optiphase 'Safe' scintillation cocktail was added, mixed thoroughly, and the radioactivity detected on LKB 1211 Minibeta scintillant counter. All results were corrected for quench and for the ^{32}P decay during the counting process.

4.2.10. INOSITIDE MODULATION

4.2.10.i Odour Stimulation

Olfactory turbinates were excised, washed and incubated in ^{32}P-orthophosphate (20-50 uCi) to label the inositides as described in section 4.2.4. After washing the tissue in cold

Ringers to remove any free orthophosphate, it was sonicated in 2 ml of Ringers for 5s (2x2.5 s, at medium power setting of the M.S.E. 100W disintegrator using a straight probe of 8mm diameter), to prepare a homogeneous suspension of labelled vesicles. The sonicate was then transferred to a centrifuge tube and centrifuged (1000xg /10 min.) to remove debris.

Aliquots (250-500 ul) of this sonicate were then added to appropriate tubes containing 2 ml Ringers and an odorant-cocktail (2 uM).The odorant-cocktail consisted of equal volumes of the following: 1so-amylacetate, (+)-carvone, anethole, m-cresol and iso-valeric acid (average mol.wt.,130; density,0.95). These were then incubated at 30°C for various times. A control sample was incubated for the same periods of time in Ringers containing no odour. Incubations were terminated by addition of 10 ml of chloroform-methanol (2:1 v/v). Extraction of phospholipids and analysis by 2D-TLC and scintillation counting were performed as described in sections 4.2.5, 4.2.6 & 4.2.9, respectively.

4.2.10.ii Activation of Phosphoinositide turnover by a G-protein

A labelled sonicate of olfactory tissue was prepared as described in section 4.2.10.i. This was challenged with buffer containing the following: GTP-γs (10-100 uM); $AlCl_3$ (100 uM), NaF (5mM). Sonicated aliquots were incubated in one of these activators for 10s at 30°C and lipids extracted and analysed

as described in sections 4.2.5, 4.2.6 & 4.2.9, respectively. A control sample was incubated in buffer containg no activator.

4.2.11 WHOLE TISSUE EXPOSURE TO ODORANT

Pairs of rat olfactory turbinates were labelled with ^{32}P as described in section 4.2.4. One half of each pair was exposed to an odorant cocktail by immersing the tissue in the head-space above the odorant cocktail in a vessel. Stimulation was for a period of 10s. The corresponding control turbinate was held in air for the same period of time. Extraction and analysis of phospholipids was carried out as described in sections4.2.5, 4.2.6 & 4.2.9, respectively.

4.2.12 EXTRACTION OF PHOSPHOLIPIDS FOR FATTY-ACID ANALYSIS

The rat olfactory tissue was prepared as described in section 4.2.3. After removal of the aqueous wash-phase 5 ml of chloroform-methanol (2:1 v/v) solution containing 0.01% BHT as the antioxidant and 0.4 ml of 1M HCL was added to the washed turbinates in a tube. The mixture was immediately sonicated, as described in section 4.2.5.i, centrifuged at 1000g for 5min, and the lower organic phase removed and washed with 1 ml of 0.3M HCl. After recentrifugation, the lower phase was filtered through phase separating paper, dried under nitrogen and stored overnight under vacuum. Special care was

taken to carry out the experiment, wherever possible, under nitrogen and in the dark in order to minimise autoxidation.

4.2.13 ISOLATION OF PHOSPHOLIPIDS FOR FATTY-ACID ANALYSIS

The isolation of phospholipids was achieved by 2D-chromatography, as described in section 4.2.6, with the exception that the developed plates were dried in an atmosphere of nitrogen, in the dark, for upto an hour. After development, the dried plates were sprayed with 0.001% (w/v) ANSA (1-amino-2-hydroxy-4-naphthalene sulphuric acid) in methanol containing 0.1% (w/v) BHT (2,6-di-tert-butyl-4-methylphenol and the major phospholipid spots visualised under ultraviolet light. In some initial experiments, developed TLC plates were sprayed lightly with 0.1% (w/v) 2.7-dichlorofluorescein in 95% (v/v) methanol containing 0.1% w/v BHT, and phospholipid spots were visualised under ultraviolet light. Minor phospholipids including the polyphosphoinositides and phosphatidic acid were located by autoradiography as described in section 4.2.7, with the exception that prior to autoradiography, the dried plates were sprayed lightly with 1% BHT in methanol, dried once again in a nitrogen atmosphere and then autoradiographed.

4.2.14 PREPARATION OF FATTY ACID METHYL ESTERS

4.2.14.i Esterification by BF_3-CH_3OH

The procedure adopted was that of Drenthe & Daemen[55] with the following modifications: The dried phospholipids isolated above, were resuspended in 40 ul chloroform-methanol(1:1, v/v) and transferred to a 'crimp-vial' containing 10 ug heneicosanoic acid (21:0) methyl ester as the internal standard. 200 ul BF_3-CH_3OH (1:7 w/v) was added, flushed with nitrogen, and the vessel sealed with a teflon rubber septum. The mixture was heated for one hour at 100°C to allow phospholipid hydrolysis and methylation of the released fatty acids to occur. After cooling the methyl esters, they were extracted by injecting 400 ul pentane into the mixture and vortexing vigorously. The top organic layer contains the methyl esters. The vials were stored at -20°C for further analysis.

4.2.14.ii Esterification by Meth-Prep II™ Reagent

The dried phospholipids were resuspended in chloroform-methanol (1:1 v/v) and transferred to a 'crimp-vial'. 200 ul Meth-Prep II™ esterification reagent (0.2N methanolic solution of [M-trifluoromethylphenyl] trimethyl ammonium hydroxide) was added, flushed with nitrogen and the vessel sealed with a teflon rubber septum. The mixture was allowed to stand at room temperature for a period of 30 mins., at the

end of which methylation of fatty acids was shown to be complete. The samples were stored st -20°C.

4.2.14.iii Esterification by H_2SO_4

The procedure followed was that adopted by Bell et al.[57], but with few modifications as described below. The fatty acyl chains of the dried-down phospholipids were transmethylated for 16 hours at 50°C under nitrogen by treatment with 2 ml of 1% (w/v) sulphuric acid in absolute methanol.

The methylation was stopped by addition of 5 ml H_2O and the crude methyl esters extracted by two washes with 5 ml hexane:diethyl ehter (1:1 v/v), containing 0.01% w/v BHT. The pooled organic phases were then extracted twice with 4 ml of 2% (w/v) $KHCO_3$ solution to remove the 2,7-dichlorofluorescein. The organic phase was dried with anhydrous Na_2SO_4 and filtered through phase separation paper and dried under nitrogen. The crude methyl esters were resuspended in hexane and transferred to a 'crimp vial', once again evaporated to dryness and then taken up in 100 ul dichloromethane containing 0.01% (w/v) BHT. The vial was flushed with nitrogen, sealed with a teflon rubber seal and stored at -20°C until further analysis.

4.2.15 ANALYSIS OF FATTY ACID METHYL ESTERS

Analysis of fatty acid methyl esters was performed in a Hewlett Packard 5890A gas chromatograph combined with a 5970 mass spectrometer (GC/MS). The GC was equipped with a capillary column, 25m in length, 0.2mm id, and coated with 0.11um film thickness of "Ultra 2" (crosslinked 5% phenyl methyl silicone). The injection port and mass selective detector were operated at 230°C and 280°C respectively. The oven temperature was programmed to increase to 200°C at a rate of 10°C/min (this temperature was retained for 6min), and then to rise by 10°C/min to a final temperature of 220°C which was retained for 12 min.

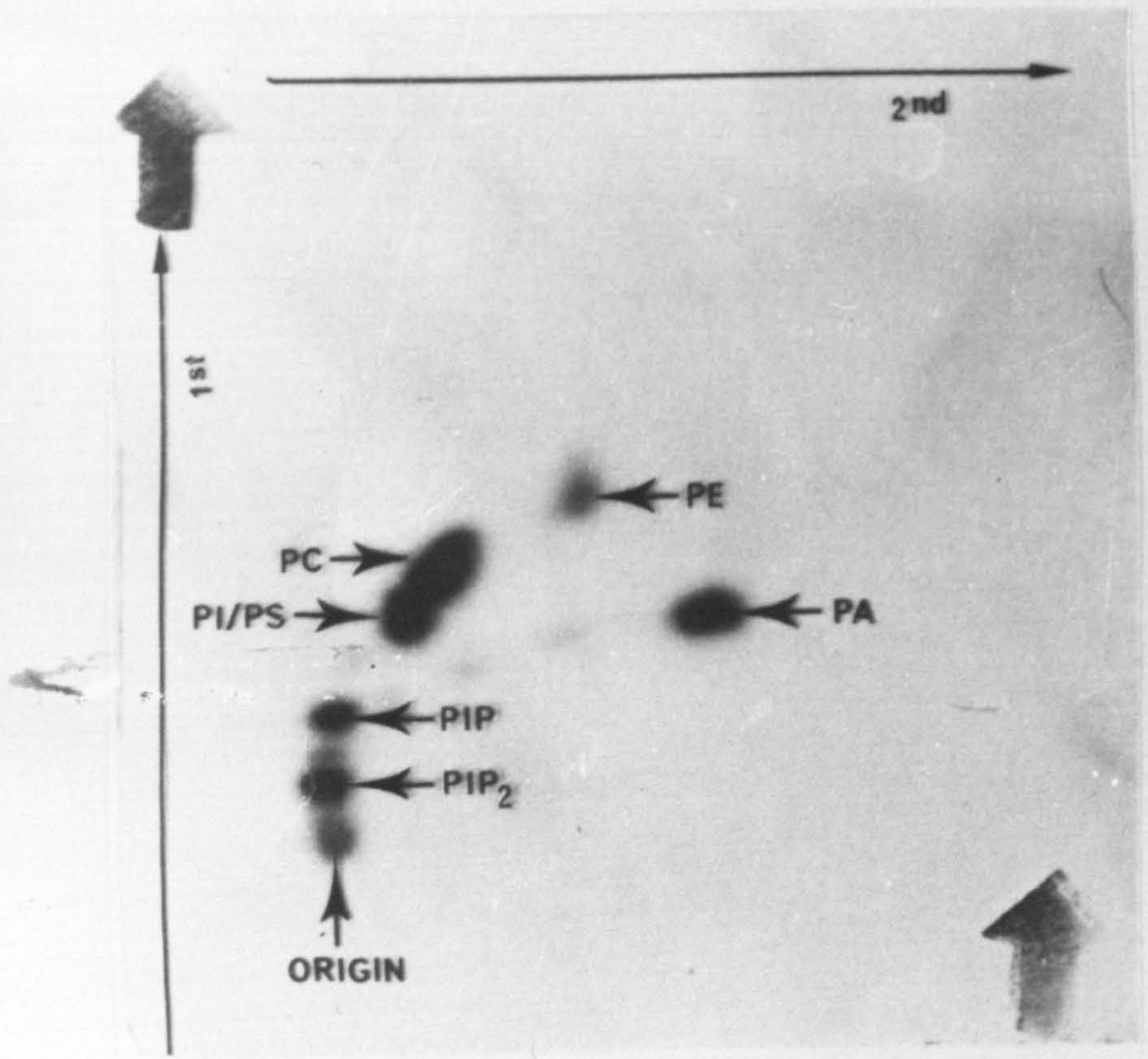
Routinely, 2ul of the methyl ester solution ,brought to room temperature, was injected into the GC. It was found necessary to concentrate the pentane extract. This was achieved by drying the extract using high purity N_2, in a fresh crimp vial. The residue was then dissolved in 10 ul of distol-grade hexane and the vial sealed. Each fatty acid methyl ester (FAME) in the extract was identified by comparing its retention time and mass spectrum with those shown by authentic FAMEs or mixtures of FAMEs. In all cases, except 22:5(n-3) methyl ester and 22:5(n-6) methyl ester, quantification of the FAME was achieved by producing a calibration graph of total ion abundance for FAME against total amount of FAME injected. The internal standard 21:0 methylester was present in all solutions at 50pmol/ul.

4.3 RESULTS AND DISCUSSION

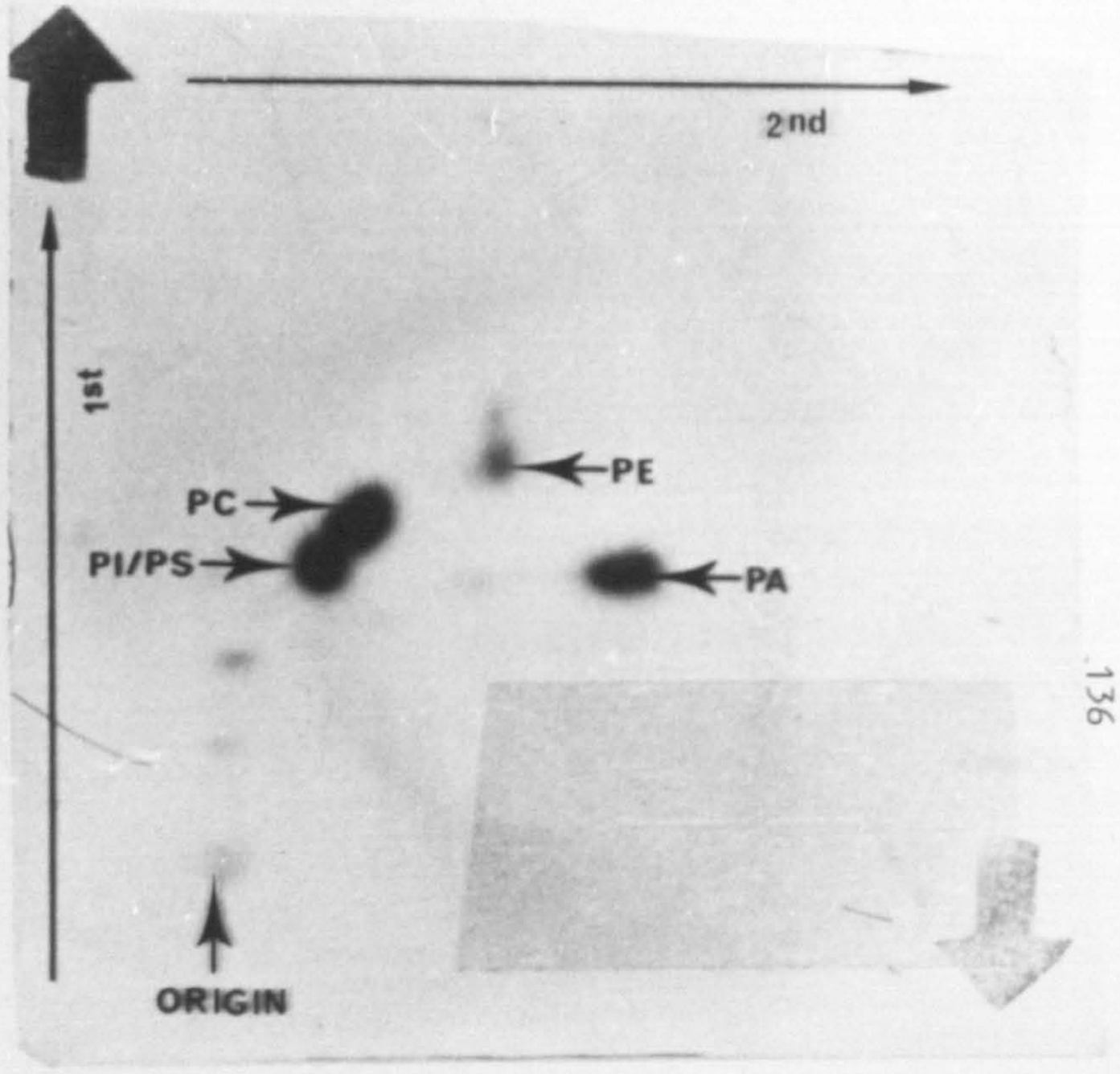
4.3.1 EXTRACTION OF PHOSPHOLIPIDS

Biochemical studies of the olfactory phospholipids were preceded by an investigation to select the best extraction and purification procedures. Two extraction procedures were examined: acid extraction and neutral extraction. Fig. 4.4 shows the autoradiographs obtained with a) acid extraction and b) neutral extraction procedures. It is apparent that a poor recovery of PIP_2 and PIP is obtained when using the neutral extraction procedure. A far greater recovery of these polyphosphoinositides is observed with an acidified aqueous wash. The major phospholipids appear to be extracted well with both extraction methods , as indicated by the intensity of the phospholipid spots on the two autoradiographs and by the concentrations of these phospholipids (see later).

The polyphosphoinositides are anionic at physiological pH, and as a consequence they are more soluble in water than in organic solvents. The five negative charges of PIP_2 enable this phospholipid to disperse in water in the form of small spherical micelles with their polar headgroups oriented outwards.[88]. The polyphosphoinositides were originally discovered as protein lipid complexes in brain tissue and, unlike other phospholipids , they were found to be insoluble in chloroform/methanol[88] mixture. Acidification of



(a)



(b)

Fig. 4.4 Autoradiographs showing two-dimensional HPTLC separations of olfactory phospholipids on layers of silica gel G, acquired by (a) acid extraction, and (b) neutral extraction procedures. Solvent system, 1st direction, chloroform-methanol-3.3 M ammonia (43:38:12 by vol.); 2nd direction, chloroform-acetone-methanol-glacial acetic acid-water (10:4:2:2:1 by vol.).

this extraction mixture with HCl is therefore required to extract the polyphosphoinositides as well as the other phospholipids[80]. The presence of acid ensures the protonation of the polyphosphoinositides and thus allows their extraction in the organic phase.

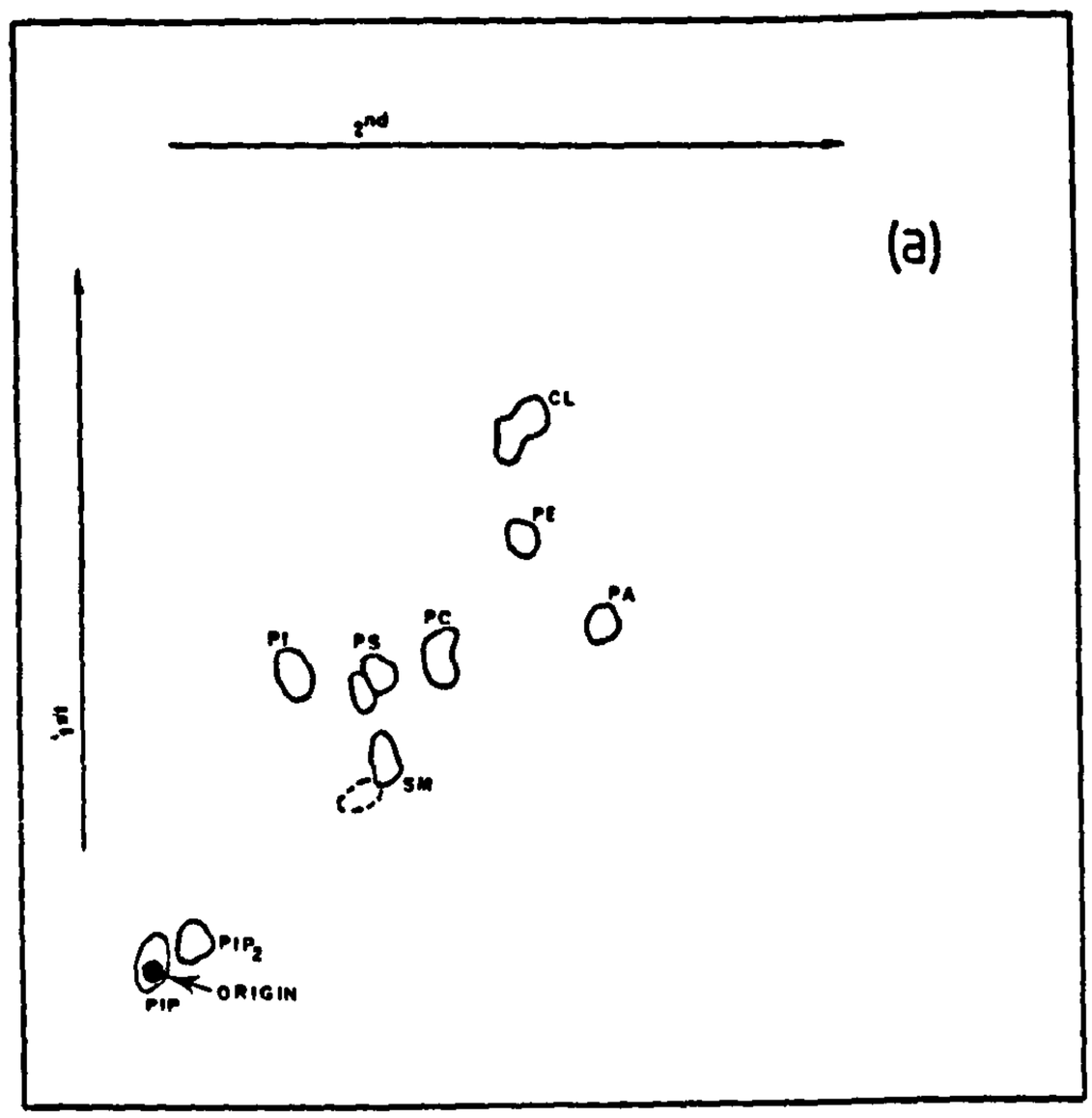
4.3.2 PURIFICATION OF PHOSPHOLIPIDS

The extracted phospholipids were purified by two dimensional chromatography on thin layers of silica gel 60, 0.25mm thick, using the following solvent systems:

(1) For the isolation of the phospholipids PA, PC, PE, PI, PS and SM, chloroform-methanol-water-ammonia sp.gr.0.88 (130:70:8:0.5 by vol.) was used in the first dimension and chloroform-acetone-methanol-glacial acetic acid-water (10:4:2:2:1, by vol.) in the second dimension (Fig. 4.5a).

(2) For the isolation of PA, PIP and PIP_2, chromatography was carried out in chloroform-methanol-3.3M ammonia (43:38:12 by vol.) in the first dimension and chloroform-acetone-methanol-acetic acid-water (10:4:2:2:1 by vol.) in the second dimension (Fig 4.5b).

It is apparent from Fig. 4.5 that excellent separation of the phospholipids is achieved. (The figure illustrates a typical separation of phospholipids; the separation obtained in different experiments were very similar, although the R_f values did tend to vary somewhat.) Care was taken not to



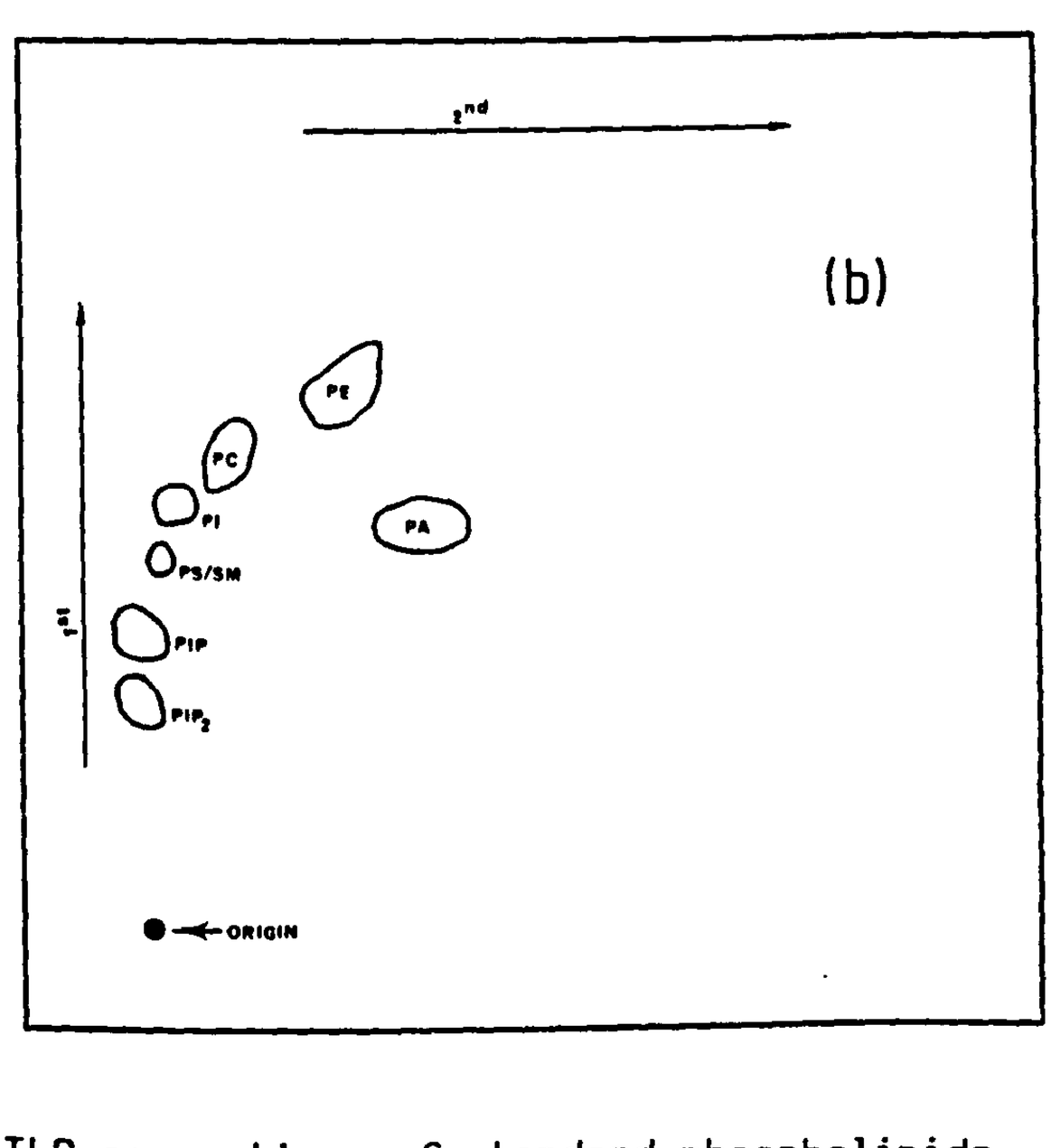


Fig. 4.5 Two-dimensional TLC separations of standard phospholipids on layers of silica gel G, using two different solvent systems.

Each phospholipid was loaded on to a 20x20 cm plate and was separated using the solvent systems (a) and (b).

(a) Solvent system: 1st direction, chloroform-methanol-water-ammonia sp. gr. 0.88 (130:70:8:0.5, by vol); 2nd direction, chloroform-acetone-methanol-acetic acid-water (10:4:2:2:1, by vol).

(b) Solvent system: 1st direction, chloroform-methanol-3.3M ammonia (43:38:12, by vol); 2nd direction, chloroform-acetone-methanol-acetic acid-water (10:4:2:2:1, by vol).

overload the plates as this caused the spots to overlap and thus reduce the spatial resolution. From preliminary experiments it was established that 100-250 ug of tissue extract was sufficient to produce a good separation. The resolution is also seriously affected by changes in temperature. It was noted that at temperatures below 20°C, separation of the phospholipid spots was very poor; the spots tended to smear in an upward direction from the origin. On cold days solvent temperatures were hence maintained at the desired temperature range of 20°C-25°C by placing the chromatography tanks in a water bath set at the appropriate temperature.

Previous studies[63] have established that in order to achieve a good separation of the polyphosphoinositides,PIP and PIP_2, the TLC plates need to be impregnated with oxalate prior to the experiment. Oxalate is thought to chelate calcium previously bound to the polyphosphoinositides thus promoting a clean separation of PIP and PIP_2 by the developing solvent systems. The effect of oxalate on the separation of the phosphoinositides was thus investigated (Fig. 4.6). Standard phospholipids were loaded onto three 10x10cm HPTLC plates coated with silica gel G 60. Plate (a) was not impregnated with potassium oxalate ; plate (b) was developed in methanol-water (2:3, v/v) containing 1% w/v potassium oxalate; plate (c) was sprayed with the above oxalate solution . Plates (b) and (c) were dried 110°C for 15min before commencing chromatography. The results illustrate that a good separation

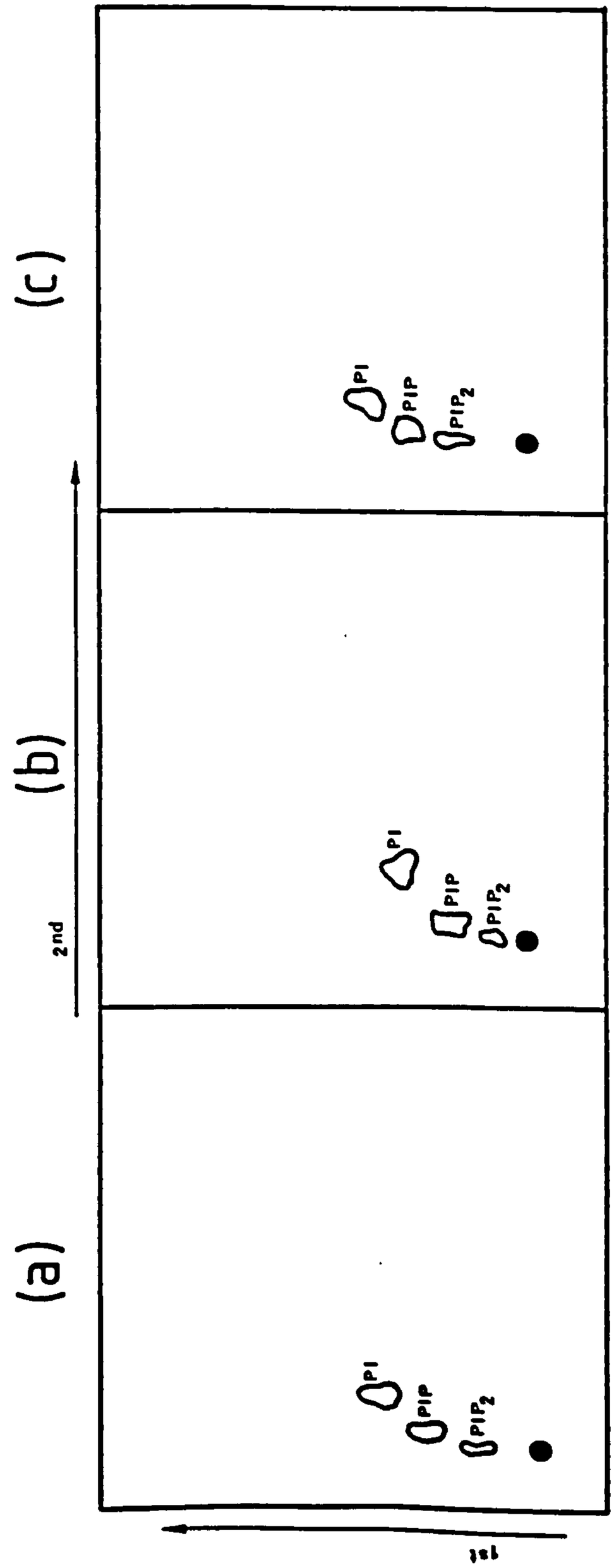


Fig. 4.6 Two-dimensional HPTLC separation of standard phosphoinositides

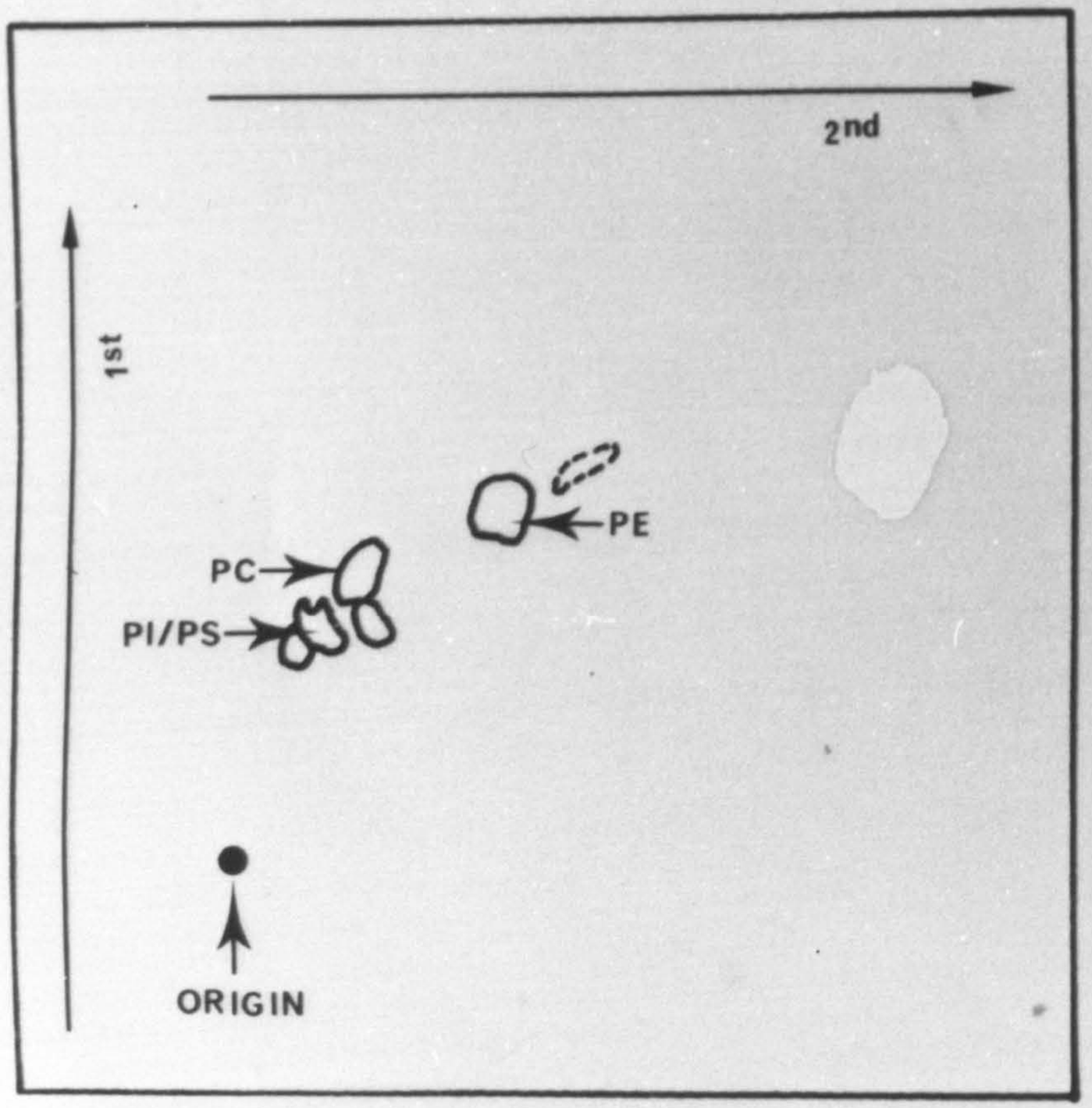
Each phospholipid (5ug) was loaded onto layers of silica gel G 10x10 cm plates. Each plate was treated differently, i.e. (a) no treatment - control, (b) oxalate-impregnated and (c) oxalate-impregnated developed plate, and sprayed plate. Solvent, 1st direction, chloroform-methanol-3.3M ammonia (43:38:12, by vol); 2nd direction, chloroform-acetone-methanol-acetic acid-water (10:4:2:2:1, by vol).

of the phosphoinositides is achieved on all three differently treated plates, contrary to the previous studies[63]. However, subsequent experiments were carried out using oxalate impregnated plates when separation of polyphosphoinositides was desired.

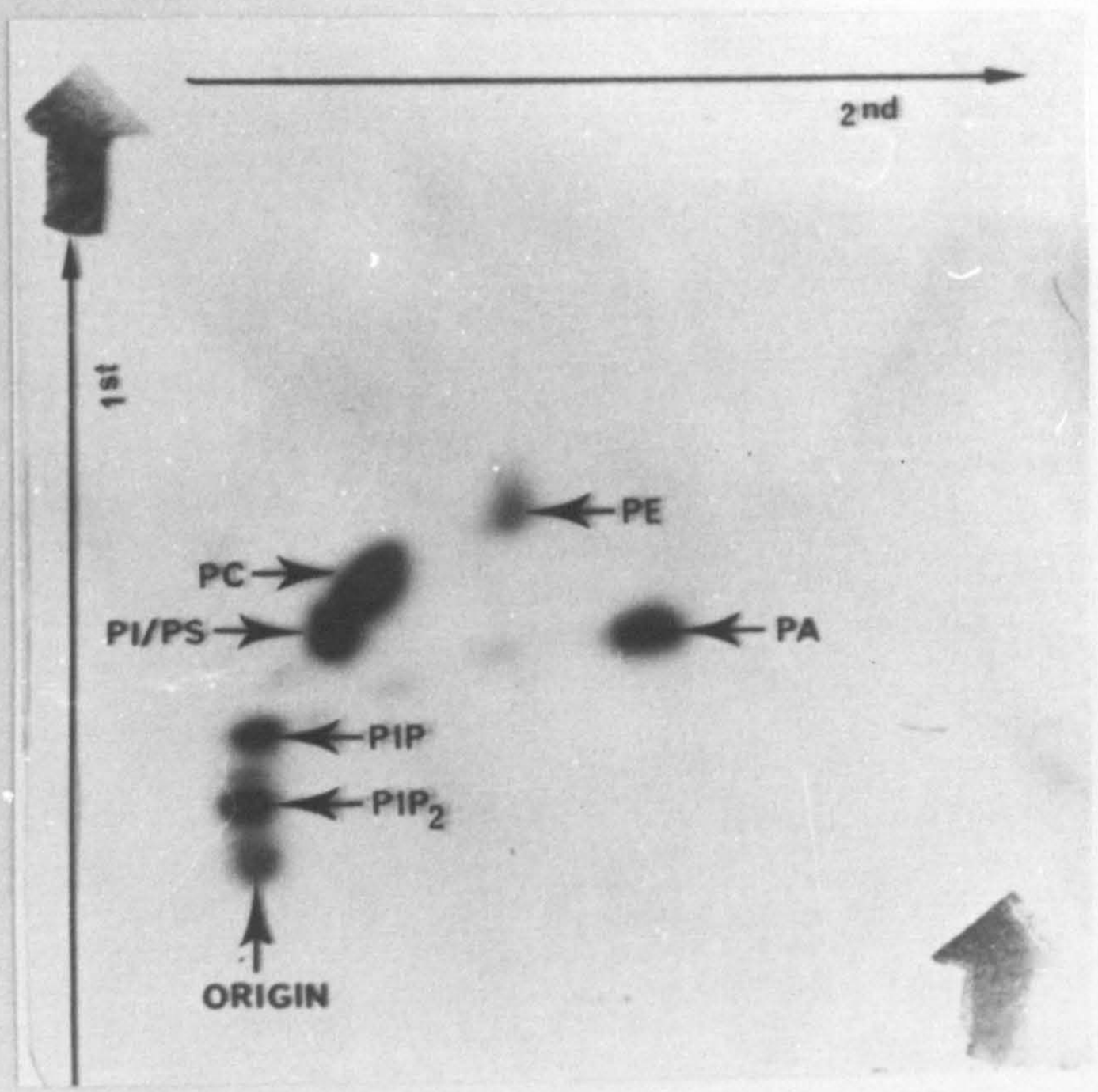
4.3.3 DETECTION OF PHOSPHOLIPIDS

Phospholipids separated on TLC plates were detected either by iodine staining or by autoradiography (Fig. 4.7). The polyphosphoinositides, PIP and PIP_2, known to be present in low concentrations in cells , are not detected by iodine vapour when the olfactory phospholipids, extracted in acidified chloroform-methanol(2:1 v/v), were separated by thin layer chromatography in two dimensions. This limitation was overcome by radiolabelling the olfactory tissue with ^{32}P orthophosphate before extracting and purifying the phospholipids, to allow their detection by autoradiography. Fig. 4.7a shows an iodine stained two dimensional separation of ^{32}P-labelled olfactory phospholipids. It may be noted that PA, PIP and PIP_2 are not visible. Fig. 4.7b shows an autoradiograph of the same thin layer plate. Here the polyphosphoinositides and phosphatidic acid spots are clearly visible on the autoradiograph. However, spots corresponding to the major phospholipids are present on both the iodine stained plate and the autoradiograph.

Polyphosphoinositides are synthesised from PI by the



(a)



(b)

142

Fig. 4.7 Figure showing (a) an iodine-stained plate, and (b) an autoradiograph of a two-dimensional HPTLC separation of olfactory phospholipids on silica gel G layers.

sequential ATP-dependent phosphorylation of the hydroxyl groups on the 4- and 5- positions of the myo-inositol moiety. This kinase-catalysed conversion of PI to its phosphorylated derivatives can be reversed by two phosphomonoesterases which specifically remove the phosphate groups from the 5- and 4- positions[4] (Fig. 4.1). The kinases and phosphomonoesterases that maintain this dynamic equilibrium between the three inositol lipids are believed to be some of the most active enzymes present in the cell[81], and they effect rapid turnover of the 4- and 5- phosphate groups of the polyphosphoinositides in many resting tissues[82]. This high rate of turnover results in the rapid transfer of ^{32}P, when added as exogenous ^{32}P orthophosphate, between inorganic phosphate, the gamma-phosphate group of ATP and the monoester phosphate groups of PIP and PIP_2. The radiolabelled phospholipids can then be readily detected by autoradiography.

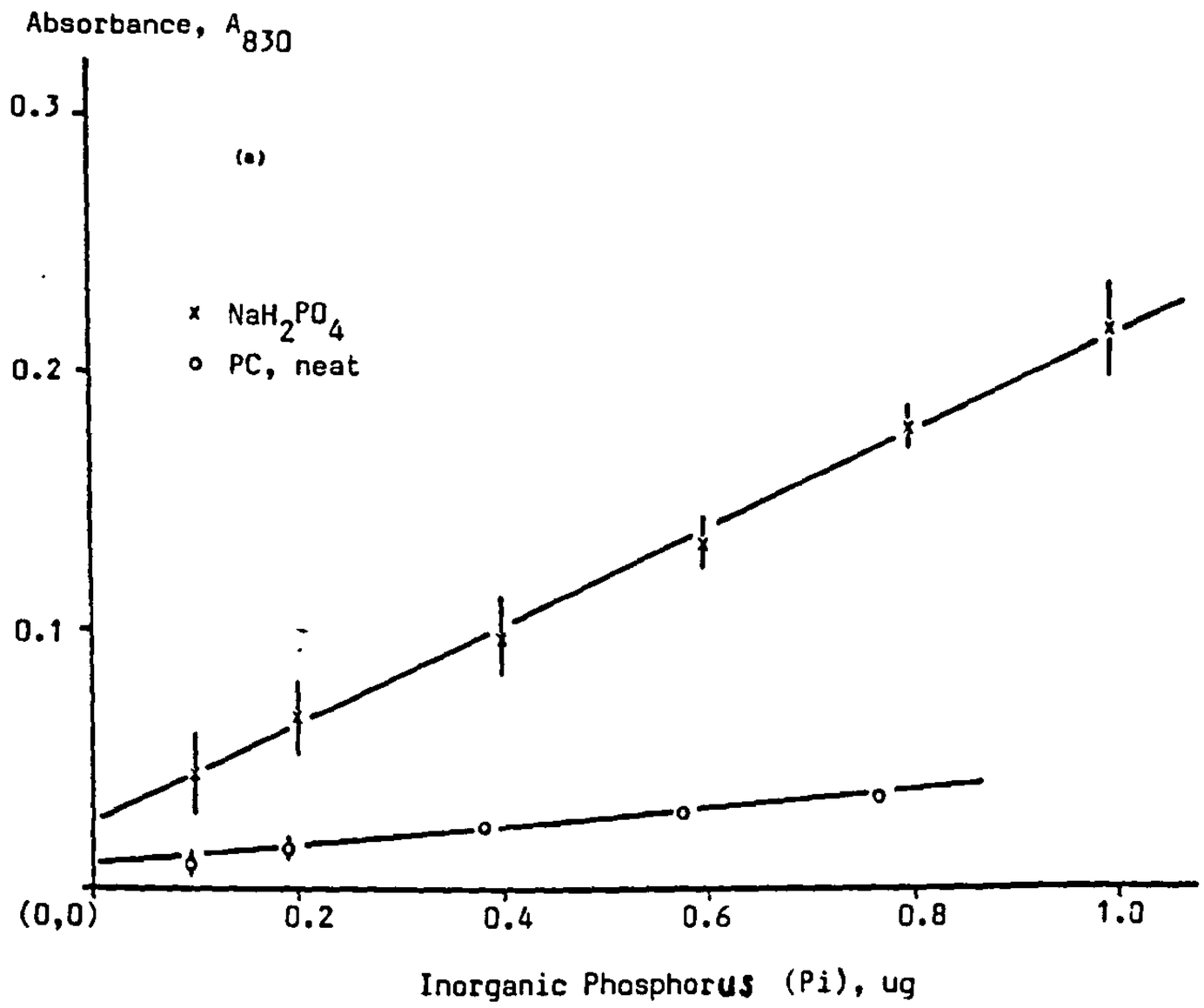
4.3.4 COMPOSITION OF PHOSPHOLIPID

Phospholipids, after separation by chromatography, were estimated by determining the amount of phosphorus in each fraction. This was achieved by following the procedures of Bartlett[84] and Pollet et al.[85] with some modifications as described in methodology. This method involved the digestion of lipid by an acid in order to convert organic into inorganic phosphorus. The inorganic phosphate was then reacted with ammonium molybdate to form phosphomolybdic acid . This was reduced and the product determined spectrophotometrically.

From preliminary experiments it was established that dry mineralisation of the samples at 300°C for a period of 21hrs was necessary for complete digestion to occur. This is illustrated in Fig. 4.8. A solution of NaH_2PO_4 (0-10ug Pi) as well as the standard phospholipid PC were used to prepare calibration curves. For Fig.4.8a, acid digestion was carried out without dry mineralisation of the samples; it was observed that very low concentrations of inorganic phosphorus are detected in the standard phospholipid PC. Hence the standard curve for PC falls well below the standard curve produced when NaH_2PO_4 is used as a standard. In Fig.4.8b , acid digestion of the samples was performed after the dried phospholipid samples were heated at 300°C in an oven for a period of 21hrs. As shown in this figure, much larger quantities of inorganic phosphorus are detected by this method. Furthermore, the standard curve and the calibration curve produced by NaH_2PO_4. are superimposable.

In order to measure the recovery of phospholipids from TLC plates, increasing concentrations of standard phospholipids (0-1 ug Pi) were chromatographed on TLC plates as usual. The phospholipid spots were detected by iodine staining and individual spots scraped off the plates and eluted in chloroform-methanol-water as described in section 4.2 above. Fig. 4.8b illustrates that an almost 100 % recovery of the phospholipid PC is obtained from silica.

For the quantification of the olfactory phospholipids



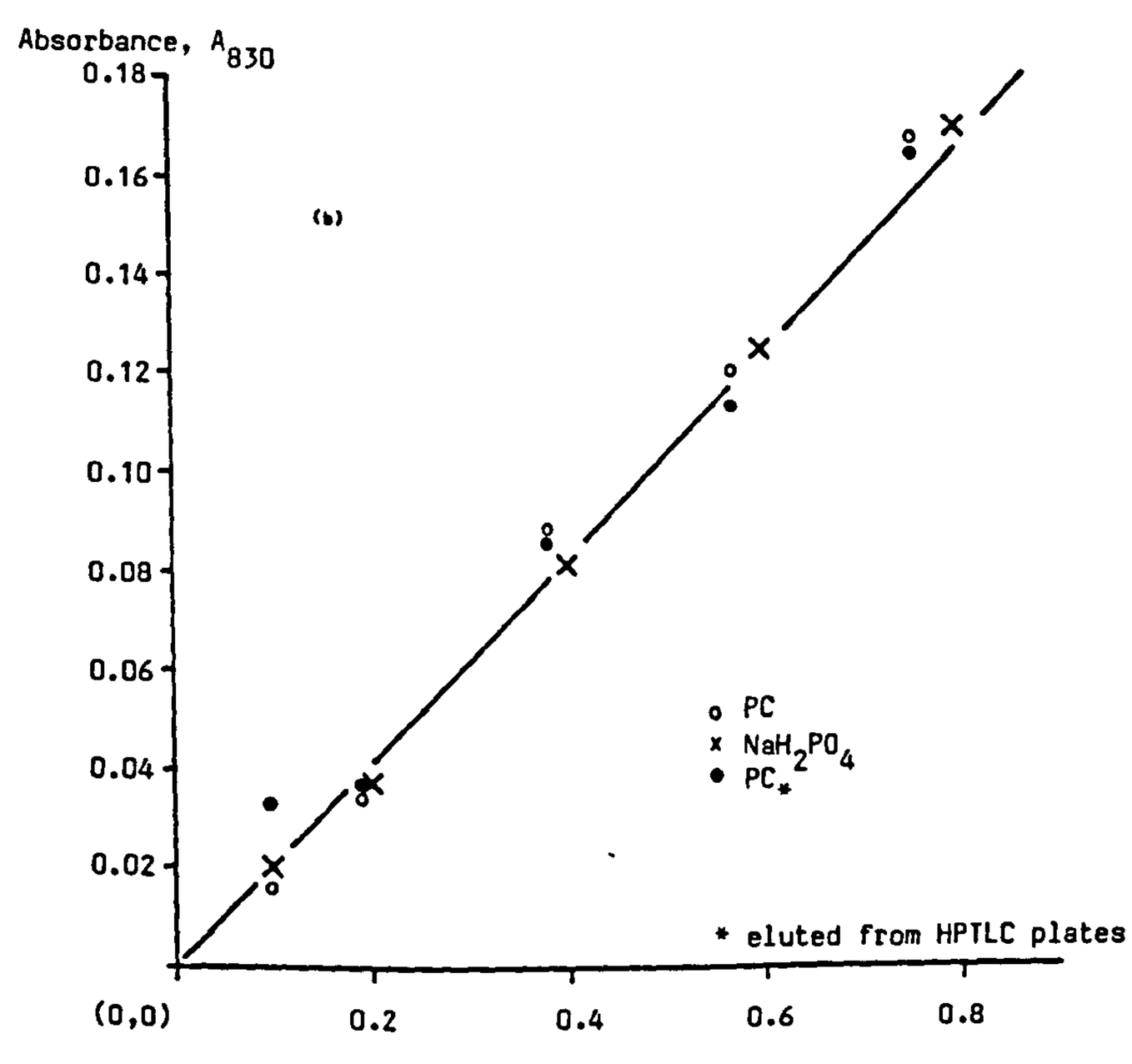


Fig. 4.8 Standard curves for phosphorus

(a) Phosphorus standard curves for NaH_2PO_4 and phosphatidyl choline (PC). The samples were digested with 70% perchloric acid to covert organic into inorganic phosphorus. Absorbance A was measured at 830 mu with a 1 cm light path. The data is expressed as the mean of duplicate determinations.

(b) Phosphorus standard curves for NaH_2PO_4, phosphatidyl choline (PC), and phosphatidyl choline eluted from silica on the TLC plates. All samples were heated at 300°C for 21 hours prior to acid digestion. Absorbance A was measured at 830 mu with a 1 cm light path. The results are a mean of triplicate determinations with a standard deviation of 5%.

it was necessary to establish whether each individual phospholipid produced a different calibration curve. If this was the case then a single experiment would require a set of calibration curves using appropriate standard phospholipids, in order to quantify each lipid. An attempt was thus made to establish whether the above was a necessity. Calibration curves were produced using a set of standard phospholipids ; these were compared with one another as well as with a standard curve obtained using NaH_2PO_4 as a standard (Fig. 4.9). The results clearly indicate that all the standard phospholipids tested released the same amounts of inorganic phosphate using the above method of dry mineralisation and acid digestion . All the standard graphs were identical to the standard curve produced by the NaH_2PO_4 solution. In subsequent experiments therefore, it was thus sufficient to use a single calibration curve produced by the standard NaH_2PO_4.

The composition of phospholipids extracted from the rat olfactory epithelium is shown in Table 4.1 and Fig. 4.10. The results are expressed as a percentage of the total phospholipid. It is evident that PC and PE are the major phospholipids of the olfactory tissue accounting for over 40% and 20%, respectively. PS, PI and SM occur in almost equal quantities representing about 10% of the phospholipid, whilst PA is present in lower amounts contributing approximately 4% to the total. PIP and PIP_2 occur in much lower quantities together representing about 4% of the total. Later results (see Fig 4.11c) show that incorporation of ^{32}P into PIP and

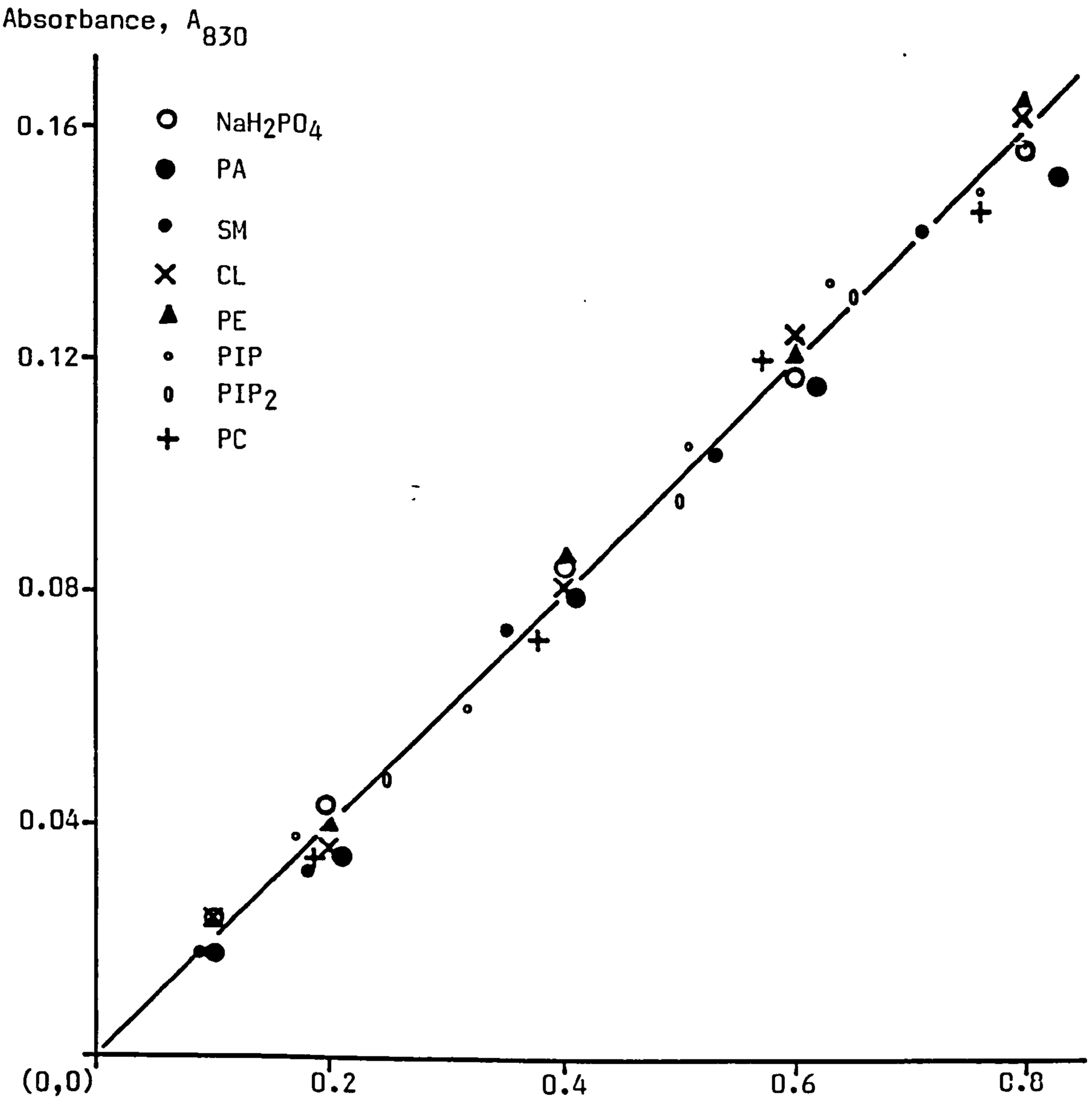


Fig. 4.9 Calibration curve for phospholipid estimation, produced routinely for inorganic phosphorus assay.
Absorbance A is measured at 830 mu with a 1 cm light path. Each point is a mean of triplicate determinations with a standard deviation of 5%.

Phospholipid	% Phospholipid / Total Phospholipid
PC	41.4 ± 4.6
PE	22.6 ± 4.9
PS	9.4 ± 3.5
SM	10.8 ± 2.0
PA	3.8 ± 1.5
PI	10.4 ± 1.9
PIP	2.4 ± 0.4
PIP_2	1.7 ± 0.5
% Total Phospholipid/ Total Crude Lipid Extract	41.5 ± 9.3

Table 4.1 The phospholipid composition of the rat olfactory epithelium.

The rat olfactory turbinates (50-100 mg) were labelled by incubating in 1 ml of Tris-buffered Ringers containing 20 uCi of ^{32}P orthophosphate for two hours at 30°C. The reaction was terminated by extracting the phospholipids with acidified chloroform-methanol (2:1, v/v) and the phospholipids separated by TLC, as detailed in Methodology. Individual spots were detected by iodine-staining and autoradiography, eluted from the plate, hydrolysed and determined as inorganic phosphate as described in section 4.2. The results are means of 4-12 separate experiments with standard deviation. (see also Fig. 4.10).

PIP_2 in the resting olfactory tissue is relatively high compared to the major phospholipids. The fact that PIP and PIP_2 are quantitatively minor components of the total olfactory tissue phospholipid, emphasises their remarkable metabolic activity. This point is illustrated in Table 4.2, where the rate of incorporation of ^{32}P is expressed as a specific activity of individual phospholipid.

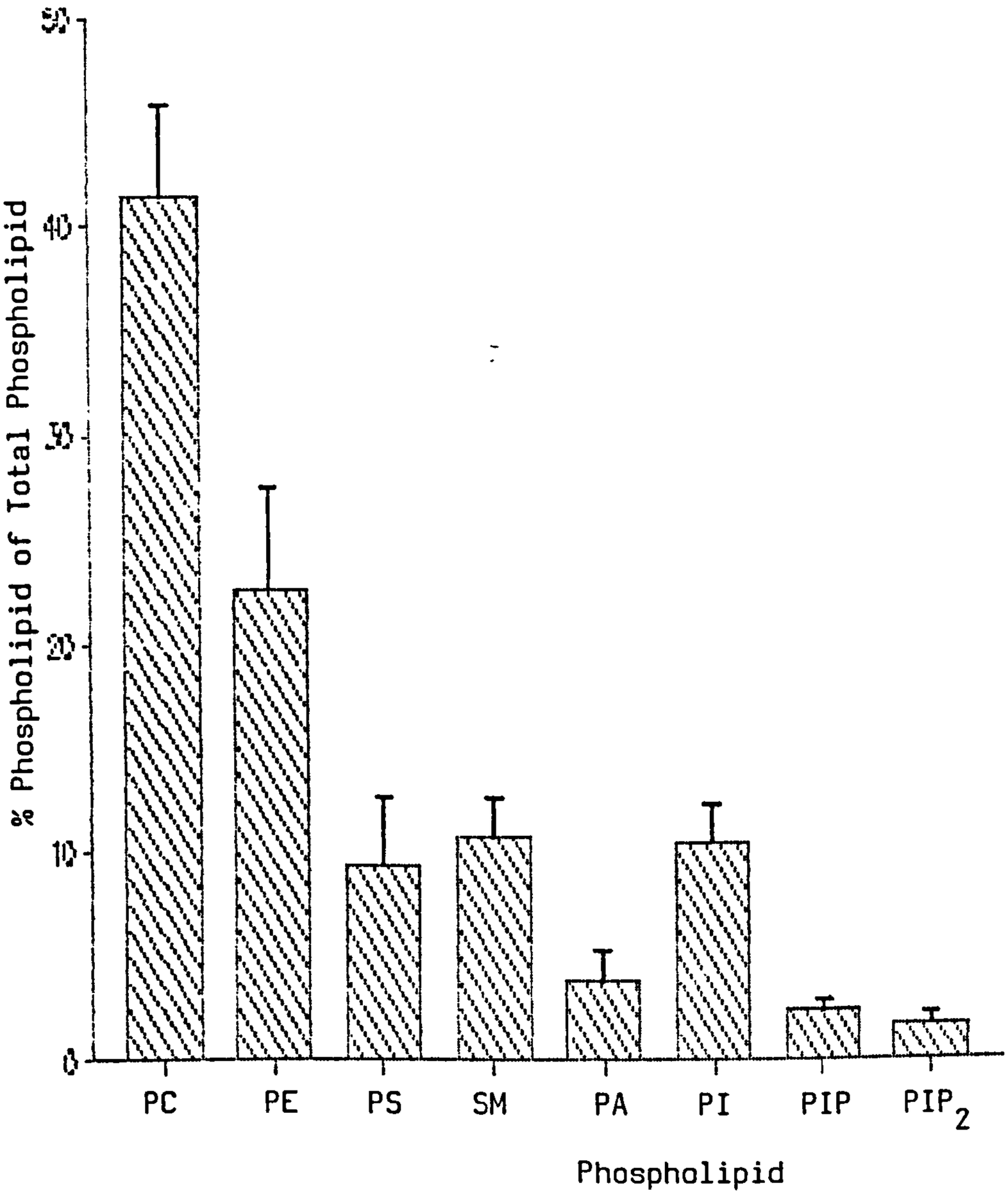


Fig. 4.10 The composition of phospholipids from the rat olfactory epithelium.

The rat turbinates were (50-100mg) were labelled by incubating in 1ml of Tris-buffered Ringers containing 20 uCi of ^{32}P orthophosphate for 2 hours at 30°C. The reaction was terminated by extracting the phospholipids with acidified chloroform-methanol (2:1, v/v) and the phospholipids were separatec by TLC as described in methodology. Individual spots were detected by iodine staining and autoradiography, eluted from the plate, hydrolysed and determined as inorganic phosphate as detailed in methodology. The results are means with standard deviation of 4-12 separate experiments.

The results in Table 4.1 also show total phospholipid expressed as a percentage of the crude lipid extract that is obtained during the extraction procedure. Total phospholipid is seen to represent approximately 40% by weight of the crude wet olfactory extract. Work was carried out in this laboratory to investigate the composition of this crude lipid extract and it was established that while approximately 40% of the extract comprised phospholipid, only approximately 10% consisted of a mixture of free fatty acids and the neutral lipids sterol, triacylglycerol, diacylglycerol, monoacylglycerol and steryl ester (Yvonne Russell - personal communication). The remaining 50% of the extract is therefore not lipid. It is thought possible that the non-lipid part of the crude olfactory extract may consist of mainly protein. It was observed that ninhydrin (known to stain the amine group), stained the origin of TLC plates where the crude extract was spotted. Taking the above findings into consideration it can therefore be said that olfactory phospholipids represent approximately 80% of total lipid in the olfactory tissue. The olfactory epithelium is therefore a rich source of phospholipids, comparable to other sensory tissues (see Table 4.3).

Phospholipid	% PL/Total PL	^{32}P Incorporation	Specific Activity
	n = 4-12	(cpm), n = 2	(cpm/% PL)*
PC	41.4 ± 4.6	10564 ± 17.7	256
PE	22.6 ± 4.9	497 ± 51.3	22
PS/SM	10.0 ± 2.7	258 ± 60.0	26
PA	3.8 ± 1.5	3252 ± 1.4	865
PI	10.4 ± 1.9	4263 ± 341.5	409
PIP	2.4 ± 0.4	3244 ± 302.6	1352
PIP_2	1.7 ± 0.5	5283 ± 243.2	3036

* Calculated from the mean values only.
± standard deviation.

Table 4.2 The rate of ^{32}P incorporation into rat olfactory phospholipids.

Rat olfactory turbinates (50-100 ug) were labelled by incubating in 1 ml of Tris-buffered Ringers containing 80 uCi of ^{32}P orthophosphate for one hour at 30°C. Reactions were terminated and individual phospholipids isolated and radio-assayed as detailed in Methodology.

Phospholipid	Tissue							
	Rat Neurons	Rabbit Neurons	Rat Brain	Mouse Brain	Rat Olfactory Tissue	Steer Tongue CVP		Rectal Gland of Dogfish
						Lateral Epidermis	Dermis	
PC	54.2	46.8	40	37	41.4	25	23.3	36.2
PE	27.1	32.2	36	33	22.6	22.4	24.6	34.9
PS	5.2	7.5	10	14	9.4	11.3	10.7	9.6
SM	4.4	7.9	7	4.5	10.8	7.5	8.4	5
PA			1	3	3.8	2.8	3.2	3.9
PI	6.0	5.6	6.0	7	10.4	9.2	9.7	9.1
PIP					2.4			1.0
PIP_2					1.7			0.9
% Total Phospholipid/ Total Lipid	72.3	74.1			80	61.0	59.3	
References	76	76	55	55	This Study	74		82

Table 4.3 The phospholipid composition of various tissues. Data is expressed as %PL/Total PL.

4.3.5 MEDIA FOR ^{32}P LABELLING

An attempt was made to find the best medium for labelling olfactory tissue with ^{32}P orthophosphate over a period of up to 5 hrs. Initial incubations were performed in bicarbonate-buffered Locke-mammalian-heart-Ringers[83], equilibriated with O_2 and 5% CO_2. This medium, consisting of NaCl (155 mM), KCl (5.6 mM), $CaCl_2$ (2.1 mM), $NaHCO_3$ (2.0 mM) and glucose (10 mM), was chosen because it is known to keep the olfactory tissue electrophysiologically active for several hours[84]. From these initial experiments it was established that the rat olfactory mucosa incorporated the radioactive isotope. An attempt was then made to optimise the incubation medium before any investigations on the phospholipase C activity were carried out. It was necessary to find a suitable substitute for the bicarbonate buffer since this buffer was not capable of maintaining pH above 7.0 when ^{32}P orthophosphate (supplied by Amersham in a solution of HCl, pH 2-6) was added to the incubation medium. The use of a phosphate buffer rather than of bicarbonate is not viable since this would create competition between the radioactive labelling of the olfactory phospholipids and that of the phosphate in the buffer, thereby reducing the sensitivity of the system. It has been established in this laboratory (see chapter 2) that a variety of organic buffers may be used with rat olfactory tissue without disrupting odorant-modulated adenylate cyclase. Thus two buffers, Hepes and Tris were investigated for their suitability (Table 4.4). The following

concentrations and pH of each buffer were calculated in order to maintain pH in the physiological range after addition of the acidic ^{32}P orthophosphate: Hepes (5.0 mM, pH 7.5), and Tris (10 mM, pH 7.8).

Buffer	Phospholipid (cpm)					
	PIP_2	PIP	PI	PA	PE	PC
Tris	1660±225	1235±14	1670±48	738±251	278±10	3165±473
Hepes	1005±77	845±190	1070±120	268±34	141±15	1557±145

Table 4.4 The effect of two different buffer systems (Tris, 10 mM, pH 7.8 and Hepes, 5 mM, pH 7.5) upon the incorporation of ^{32}P orthophosphate into phospholipids.

Rat olfactory turbinates (50-100 mg) were incubated in Ringer-based media, buffered with either Tris or Hepes, containing 80 uCi of ^{32}P orthophosphate, at 30°C for 90 min. Reactions were terminated and phospholipids isolated, and radio-assayed as detailed in Methodology. The results represent the amount of radioactivity (cpm) incorporated into each phospholipid and are means of duplicate determinations with standard deviation.

Table 4.4 shows the effect of these two buffers upon the amount of ^{32}P incorporated into each phospholipid. The results indicate that both buffer systems support the incorporation of ^{32}P into the olfactory phospholipids. The Tris buffered tissue, however, shows slightly higher overall activity in each phospholipid. Furthermore, Tris is a smaller molecule than Hepes and therefore is less likely to behave as an odorant. In view of these factors, subsequent experiments were performed in Tris buffered media.

Table 4.5 demonstrates the effect of the presence or absence of glucose upon the rate of incorporation of ^{32}P orthophosphate into olfactory tissue. It was thought possible that the glucose present in the system may behave as an odorant or may have some other kind of stimulatory effect. (The presence of sucrose was observed to have a stimulatory effect on the adenylate cyclase activity, see Chapter 2). The above experiment was hence performed in an attempt to establish whether the olfactory tissue was capable of incorporating ^{32}P in the absence of glucose in the incubation medium. The results in Table 4.5 indicate that glucose does not significantly affect the incorporation of the radioactive isotope into the olfactory tissue. Therefore, the presence of glucose is not a requirement in the incubation medium. Subsequent experiments were then performed in the following medium: Tris-HCl (10 mM, pH 7.8), NaCl (155 mM), KCl (5.6 mM) and $CaCl_2$ (2.1 mM).

Glucose (mM)	Phospholipids (cpm)					
	PIP_2	PIP	PI	PA	PE	PC
0	610	450	575	300	100	1836
10	690	355	400	230	85	970

Table 4.5 The effect of glucose upon the level of ^{32}P orthophosphate incorporation into olfactory phospholipids.

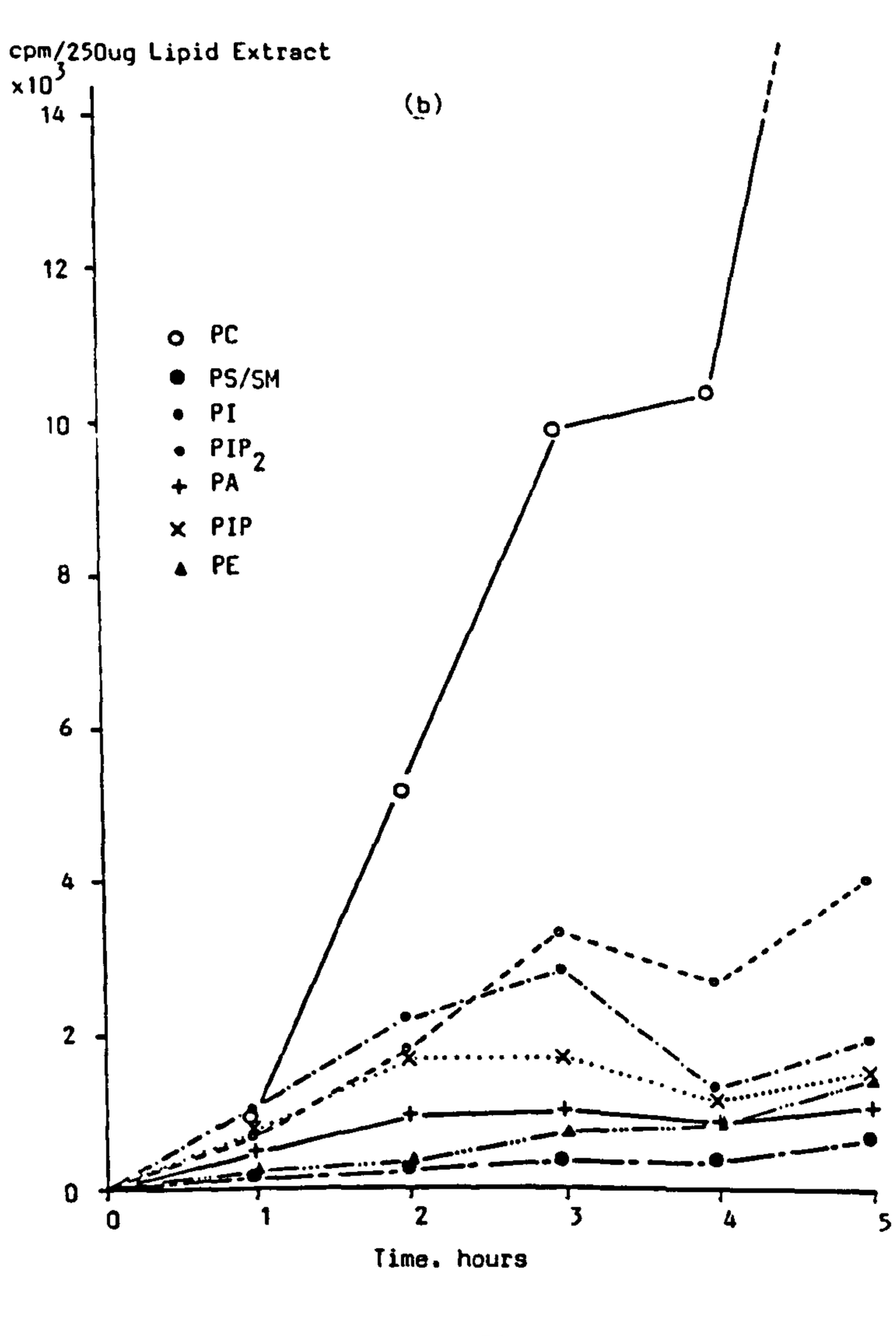
Rat olfactory turbinates (50-100 mg) were labelled by incubating in Tris-buffered Ringers (+/- glucose) containing 80 uCi ^{32}P orthophosphate at 30°C for 90 min. Reactions were terminated and phospholipids isolated and radio-assayed as detailed in Methodology. The results are expressed as the amount of radioactivity (cpm) incorporated into each phospholipid and are means of three replicates in a single experiment with a standard deviation of 5%.

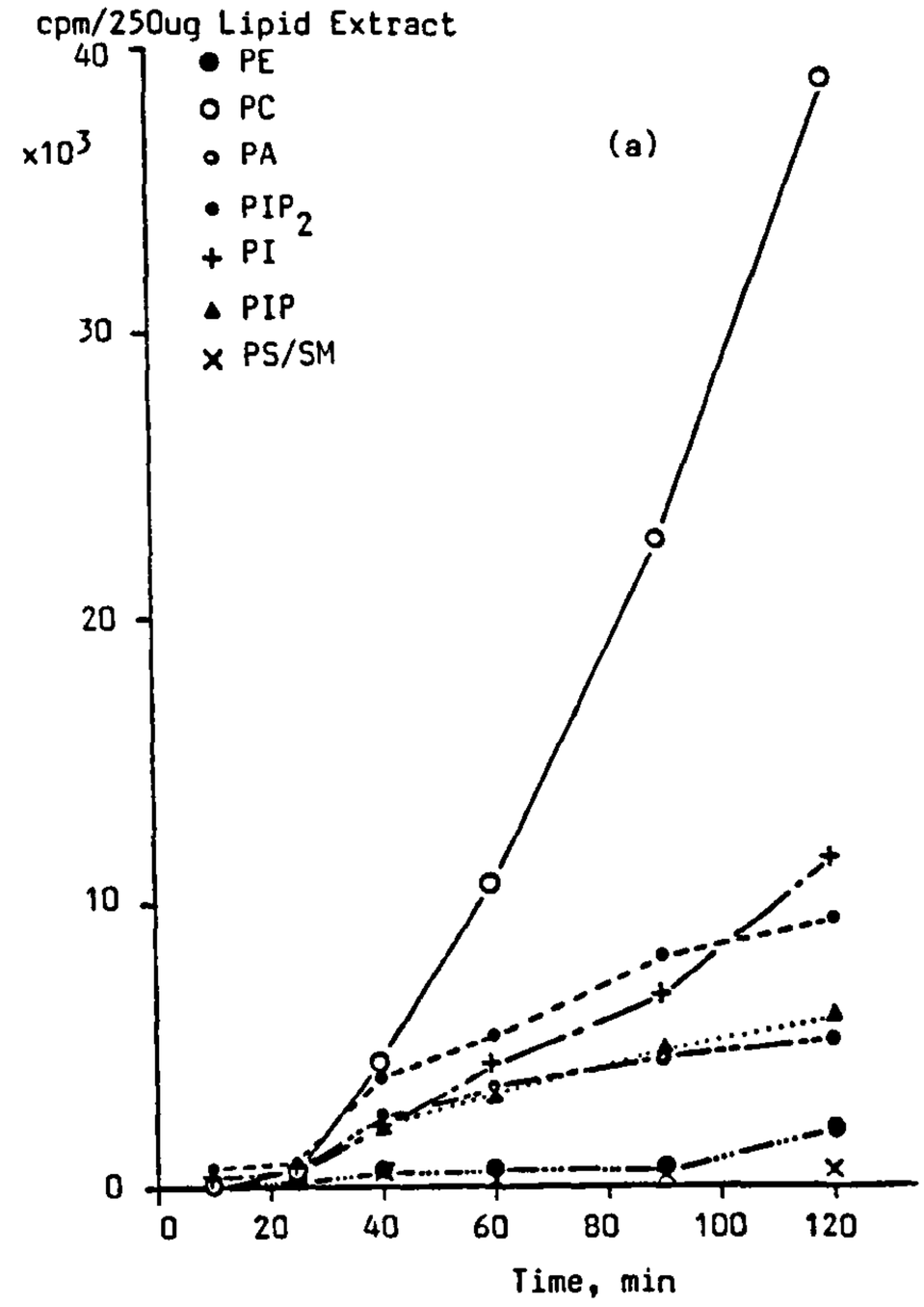
4.3.6 RATE OF ^{32}P ORTHOPHOSPHATE INCORPORATION INTO OLFACTORY PHOSPHOLIPIDS.

The metabolic activities of the polyphosphoinositides in the resting olfactory tissue were studied by incubating olfactory turbinates in Ringer solution in the presence of ^{32}P orthophosphate, and following the uptake of label into lipids. In order to find the optimum duration for incubation of rat olfactory tissue, a series of time-course experiments was performed (Fig. 4.11), where the relative rates of incorporation into each phospholipid were measured. In initial time-course experiments, for each rat, half the tissue was incubated for a "sample time" varying from 10 to 120 min., whilst the other half was incubated for a constant time of 90 min. for each rat. In this way, it was hoped that some of the inter-rat variation could be normalised by presenting data as a function of the control time. This approach worked well when the sample time was much smaller than the control time. However, as the sample time approached the control time, the results became rather variable, presumably because of variations in metabolic rates from tissue obtained from the same animal. Eventually, a procedure was adopted in which each pair of turbinates from an animal were incubated for the same length of time to provide duplicates. The first time-course experiment performed in this way involved six rats and followed incorporation up to 120 min. As no plateauing of the incorporation curves was apparent after 120 min, a second study was performed following the incubation over a five hour

period. The results of the two incorporation studies are shown as plots of cpm/time in Fig. 4.11a & b, respectively. Certain trends in the pattern of incorporation are immediately apparent. Firstly, PC incorporates at the greatest rate; the general pattern of incorporation rates is PC >> PI, PIP, PIP_2 and PA > PS, PE, and SM. From this it appears that PC is the most metabolically active phospholipid in the rat olfactory mucosa. However, if the relative abundance of each phospholipid is taken into account and a plot of relative specific activity against time is made, it becomes apparent that the inositides are incorporating to a much higher specific activity than the other phospholipids (Fig. 4.11c). The higher rates of incorporation of ^{32}P orthophosphate into PIP and PIP_2 presumably reflect a rapid rate of turnover of the phosphate groups in the 4- and 5-positions. These results therefore indicate that in the olfactory tissue, the polyphosphoinositides are metabolically active in the resting tissue.

From Fig. 4.11c it is also apparent that the rate of incorporation of ^{32}P orthophosphate into phospholipids rises steadily over a period of 3 hours. Longer incubation periods seem to show a small change in the rate, indicating a possible onset of steady-state conditions.





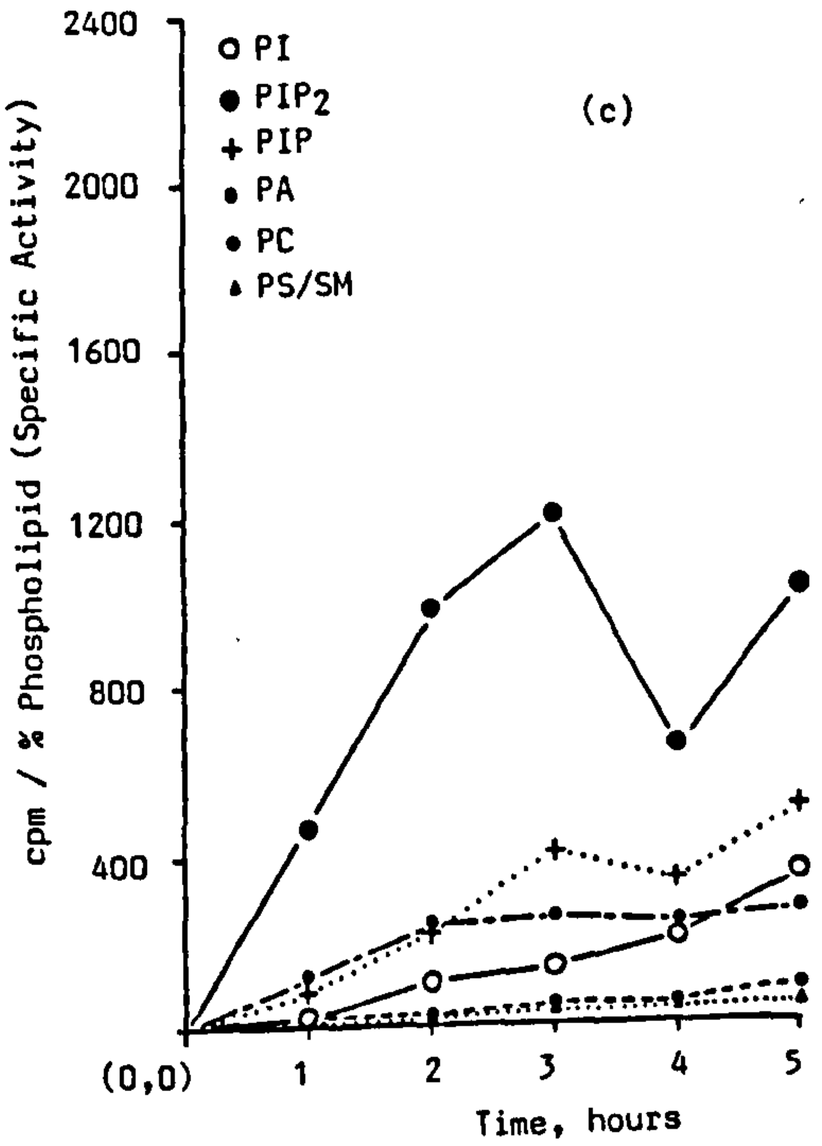


Fig. 4.11 The incorporation of ^{32}P orthophosphate into rat olfactory phospholipids.

(a) & (b) Rat olfactory turbinates (50-100mg) were incubated in Tris-buffered Ringers (1.0ml) containing (a) 80 uCi and (b) 20 uCi of ^{32}P orthophosphate at 30°C for up to (a) 2 hours and (b) 5 hours. Reactions were terminated and individual phospholipids isolated and radioassayed as described in methodology. The data represent means of duplicate determinations with a standard deviation of 7%.

(c) The data is expressed as the relative specific activity against time. Experimental conditions are those described for graphs (a) and (b) above.

4.3.7 MODULATION OF PHOSPHOINOSITIDE TURNOVER

Having established that there is rapid metabolism of inositol phospholipids in the olfactory mucosa, a series of experiments aimed at finding agonists that may modulate the level of these lipids were performed (Tables 4.6, 4.7 and 4.8).

4.3.7.i The effect of an odour

As the inositides represent a transduction mechanism and, in this instance, are found in a tissue concerned specifically with odour recognition, the effect of an odour was investigated. The odorant activation experiments were performed on radiolabelled sonicated olfactory tissue as well as on a whole tissue preparation. When the sonicated preparation was used, material from one rat was divided into four or more aliquots. It was thus desirable to increase sensitivity by labelling the tissue to a higher specific activity than was used in the ^{32}P incorporation studies above. Hence, the tissue was usually incubated in 50 uCi/ml of ^{32}p orthophosphate. The results are shown in Tables 4.6 and 4.7. The data have been normalised with respect to PC (the large number of counts incorporated into PC serves as a useful internal marker and therefore can be used to account for any variation in the quantity of tissue aliquoted). For this, however, the assumption is made that labelled PC in the preparation does not vary significantly in the course of an

experiment.

	Phospholipid (cpm)					
	PIP_2	PIP	PI	PA	PE	PC
Basal Level*	600	275	2701	1038	636	6183
Stimulated• Level	623	314	2825	1058	647	6183
% Stimulation	3.8	14	4.6	1.9	1.7	-

* No odour; • With odour

Table 4.6 The effect of odour on the rate of incorporation of ^{32}P orthophosphate into olfactory phospholipids.

Rat olfactory turbinates (50-100 mg) were pre-labelled in Tris-buffered Ringers (1 ml) containing 50 uCi of ^{32}P orthophosphate at 30°C for 2 hours. Aliquots of the supernatant from the sonicated preparation were incubated in Tris-buffered Ringers, in the presence or absence of odorant "cocktail", at 30°C for 10 min. Reactions were terminated and the phospholipids isolated and radio-assayed as detailed in Methodology. The results are expressed as the amount of radioactivity (cpm) incorporated into each phospholipid and represent means of three replicates in a single experiment with a standard deviation of 5 %.

If rat olfaction was mediated in part by inositol lipids, then the presence of an odour would be expected to stimulate inositide metabolism, thereby causing the breakdown of the polyphosphoinositides. Thus, in the above experiment levels of radioactivity incorporated into PIP and PIP_2 would be expected to deplete rapidly after stimulation by an odour. However, the results show no such changes (see Table 4.6). Creba et al.[88] in 1983 demonstrated the stimulatory effect of vasopressin on rat hepatocytes. They established that upon vasopressin stimulation of pre-labelled cells, there was a

rapid fall in the labelling of PIP and PIP_2, which reached a maximum in 1-2 min. They also reported that after about a 2 min. period, there was a recovery of the polyphosphoinositide labelling towards the initial pre-stimulation levels. Similarly, Thomas et al.[66] also working on isolated hepatocytes, reported that vasopressin stimulation caused approximately a 50 % depletion of PIP and PIP_2 labelling within 10 seconds of stimulation, and that these levels began to recover to their initial values after 30 seconds.

These findings may therefore explain the lack of stimulation seen in the odour modulation experiment described above. It is possible that the 10 min. stimulation period used above is too long. If changes in the levels of inositides occur rapidly, then the above approach of monitoring these levels is not likely to pick up the response before the initial levels are restored.

The effect of shorter stimulation periods (2,10 and 60s) on the rate of ^{32}P incorporation into olfactory tissue was then investigated (Table 4.7). Some perturbations in the levels of radioactivity incorporated into PIP and PIP_2 are observed relative to the control tissue. These changes, however, are small and more experiments are required to substantiate these observations. The results show that after a 2 s odour stimulation, the levels of labelled PIP_2 fall by ca. 35 %; PIP levels deplete by approximately ca. 34 %, and much smaller changes occur in the labelling of the remaining

Stimulation Time (sec)	Phospholipids (cpm)					
	PIP_2	PIP	PI	PA	PE	PC
0	164	126	441	268	70	1659
2	106	83	460	242	76	1780
% Change	-35.4	-34.1	4.3	-9.7	8.6	7.3
10	102	84	465	238	76	1803
% Change	-37.8	-33.3	5.4	-11.2	8.6	8.7
60	114	88	403	243	83	1735
% Change	-30.5	-30.2	-8.6	-9.3	18.6	4.6

Table 4.7 Time course of the effect of odour on the rate of incorporation of ^{32}P orthophosphate into olfactory phospholipids.

Rat olfactory turbinates (50-100 mg) were pre-labelled in Tris-buffered Ringers (1 ml) containing 50 uCi of ^{32}P orthophosphate at 30°C for 2 hours. Aliquots of the supernatant from the sonicated preparation were incubated in Tris-buffered Ringers in the presence or absence of odorant "cocktail" at 30°C for the indicated periods of time. Reactions were terminated and the phospholipids isolated and radio-assayed as detailed in Methodology. The results are expressed as the amount of radioactivity (cpm) incorporated into each phospholipid and normalised with respect to total number of counts obtained in the control tissue. Percentage change is shown relative to the pre-stimulated levels. Data represent means of three replicates in a single experiment with a standard deviation of 5 %.

phospholipids. After a 10 s odour stimulation, the levels of labelled PIP_2 and PIP appear to have steadied, showing an approximately 37 % and 33 %, respectively, decrease from the non-stimulated levels. The levels of the remaining phospholipids once again show relatively no large change. After a 60 s odour stimulation period the results seem to indicate that the levels of labelled polyphosphoinositides are

slowly recovering. The PIP and PIP_2 levels are found to be ca. 31 % and ca. 30 %, respectively, of the initial pre-stimulated levels. The remaining phospholipids once again do not indicate much change.

The above results, hence, seem to indicate that some changes do occur in the levels of the olfactory inositides after stimulation of the tissue by an odour. These observations suggest that odour stimulation causes rapid breakdown of the polyphosphoinositides within 2 s, followed by a much slower re-synthesis of the PIP and PIP_2 towards pre-stimulated levels. The depletion and apparent recovery of these polyphosphoinositides appears to follow a similar pattern to that reported by Creba et al.[88] and Thomas et al.[88].

The results obtained from a whole tissue activation are shown in Table 4.8. In this experiment, direct tissue activation was achieved by exposing half the olfactory tissue from one animal to the odour vapour for a period of 10 s and leaving the other half of the tissue unchallenged. This method has certain disadvantages. There is considerable inter-animal variation which therefore demands that large numbers of animals be used in order to ensure statistical reliability. This approach also requires one animal to be used for each data point which further increases the total number of animals required for a given experiment.

	Phospholipids (cpm)					
	PIP_2	PIP	PI	PA	PE	PC
Basal Level*	1335±8	1050±20	1768±47	1257±59	315±18	1610±29
Stimulated• Level	1465±23	1364±29	1555±18	1077±13	314±25	1581±0.7
% Stimulation	9.7	29.9	-12.0	-14.3	-0.3	-1.8

* No Odour; • With Odour

Table 4.8 The effect of a whole tissue exposure to odorant on the rate of ^{32}P orthophosphate incorporation into olfactory phospholipids.

Rat olfactory turbinates (50-100 mg) were pre-labelled by incubating in Tris-buffered Ringers (1 ml) containing 50 uCi of ^{32}P orthophosphate at 30°C for 2 hours. Reactions were terminated and the tissue was exposed to odour vapour for a period of 10 s. Control tissue was held in air for the same period of time. Reactions were terminated and the phospholipids isolated and radio-assayed as described in Methodology. The results are expressed as the amount of radioactivity (cpm) incorporated into each phospholipid and normalised with respect to total number of counts obtained in the control tissue. Percentage stimulation is shown relative to the pre-stimulated levels. Data represent means of two experiments with standard deviation.

The results obtained conflict with the data from the sonication experiments above (Table 4.7). The levels of labelled PIP_2 and PIP appear to rise by approximately 10 % and 30 % respectively, after a 10 s stimulation by an odour. The levels of the remaining labelled phospholipids appear to decrease.

From the odour modulation experiment reported above, it is apparent that many more experiments need to be carried out in order to substantiate the observations made. One can,

however, conclude that the presence of an odour has some effect on the polyphosphoinositides within the olfactory tissue. What this effect truly is has yet to be verified. Furthermore, it is interesting to note that the major phospholipids seem to be affected to a lesser degree by the odour compared to the polyphosphoinositides.

4.3.7. ii The effect of GTPγS and AlF_3

It is now widely accepted that agonist-stimulated phosphoinositide metabolism is mediated by guanine nucleotide-binding proteins[32,33] (see Fig. 4.3). This has been demonstrated by the ability of GTP analogues such as GTPγS and other activators such as AlF_3 to stimulate inositide metabolism. It is believed that this is brought about by interaction of the activated G-protein with the phosphoinositide-specific phospholipase C (phosphoinositidase C or PIC). In an attempt to demonstrate the presence of PIC activity in the olfactory preparation, a labelled sonicate of olfactory tissue was challenged with buffer containing GTPγS or AlF_3 as described in section 4.2.

Table 4.9 shows the results from an attempt to activate PIC using G-protein activators. No significant change in the levels of any lipid is observed.

	Phospholipids (cpm)					
	PIP_2	PIP	PI	PA	PE	PC
Control	1391±11	964±7	3147±17	961±52	965±33	8769±20
GTP 10 uM	1390±7	972±40	3402±84	963±20	844±4	8626±52
% Change	0	0.8	8.1	0.2	-12.5	-1.6
GTP 100 uM	1334±30	1039±90	3331±78	940±62	815±42	8739±170
% Change	-4.1	7.8	5.9	-2.1	-15.5	-0.3
AlF_3	1375±29	923±12	3328±7	921±17	878±68	8772±36
%Change	-1.1	-4.3	5.8	-4.2	-9.0	0

Table 4.9 The effect of $GTP\gamma S$ and AlF_3 on the rate of ^{32}P orthophosphate incorporation into olfactory phospholipids.

Rat olfactory tissue was pre-labelled with ^{32}P orthophosphate in the usual way for a period of 2 hrs. Aliquots of the sonicated supernatant were incubated in the presence or absence (control) of the indicated compounds for a period of 10 s at 30°C. Reactions were terminated and the phospholipids isolated and radio-assayed as detailed in Methodology. The results are expressed as the amount of radioactivity (cpm) incorporated into each phospholipid and normalised with respect to total number of counts obtained in the control tissue. Percentage change is shown relative to the pre-stimulated (control) levels. Results are means of two separate experiments with standard deviation.

4.3.8 FATTY ACID ANALYSIS

Phospholipids in the biomembranes of terrestrial mammals contain fatty acids mainly in the chain length range of 16-20, the major acids being palmitic (16:0), stearic (18:0) and oleic (18:1 [n-9]), linoleic (18:2 [n-6]) and arachidonic (20:4 [n-6])[87]. In terrestrial mammals, phospholipids are characteristically rich in (n-6) polyunsaturated fatty acids[88] and PI is not only an intermediate in the inositol phospholipid cycle but also serves as a source of arachidonic acid (20:4 [n-6]), the precursor of prostaglandins E_2 and $F_{2\alpha}$[88].

The degree of unsaturation of the fatty acids is important in determining the fluidity of the membrane and in providing the correct environment for membrane functions. Interest in the possible role of phospholipids and fatty acids in olfaction prompted an analysis of the fatty acid composition of phospholipids from the olfactory turbinates.

An attempt was made to find a suitable method for preparing fatty acid methyl esters of olfactory phospholipids. Three different methods were used to prepare methyl esters of fatty acids as described above, i.e. esterification by BF_3-MeOH, Meth. Prep. II™ reagent and H_2SO_4. Fig. 4.12 illustrates the fatty acid profiles present in the crude olfactory lipid extracts obtained, prior to purification and separation of phospholipids by TLC. The results show that all

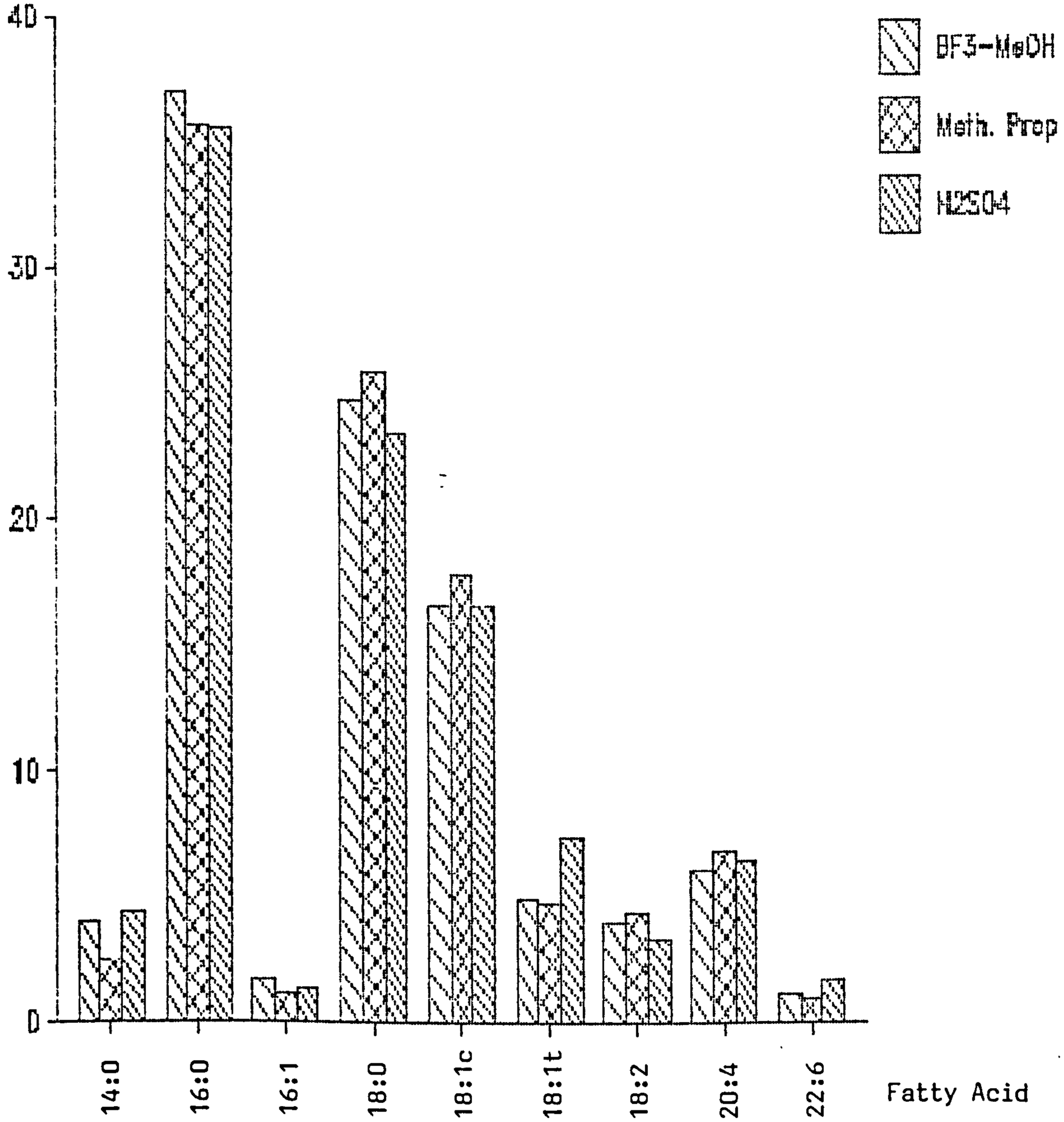


Fig. 4.12 Fatty acid analysis of Methyl esters from the crude olfactory lipid extract, using three different mediums: BF_3-MeOH, Meth. Prep II™ and H_2SO_4.

The rat olfactory turbinates (50-100mg) were used. The lipids were extracted with acidified chloroform-methanol (2:1, v/v). Fatty acid methyl esters of the crude extract were analysed by GC/MS as detailed in methodology. The results are expressed as wt% and represent the data from a single experiment.

three methods produced similar profiles, the identified fatty acids being present in similar quantities. However, the TIC trace (total ion chromatogram) indicated that the BF_3-MeOH method produced the "cleanest" preparation in that the TIC peaks were clear and easily identified. The acid-catalysed esterification method produced large impurity peaks that made analysis difficult and tedious. Similarly, the method using the Meth. Prep. II™ reagent also resulted in methyl esters that contained many impurities. BF_3-MeOH was thus used in subsequent experiments to prepare methyl esters of olfactory phospholipids for the analysis of fatty acids.

Table 4.10 and Fig. 4.13 details the type and concentration of fatty acids present in the olfactory phospholipids PI, PS, PC, PE and SM. The results are expressed as percentages of total fatty acids present and were calculated as described in section 4.2. The values shown are means of 5-8 separate experiments. It is apparent that the standard deviations are rather high, possibly due to inter-rat variation, i.e. each separate experiment involved extraction of phospholipids from a single animal. The fatty acid distribution in the olfactory tissue from these rats show a large variation, as may be expected, since factors such as diet and age of the animal could affect the fatty acid compositions[70]. The animals used for experiments were given food and water as normal without control in diet. This factor may prove to be an important one and needs to considered in the future.

% TOTAL FATTY ACID (f.a.)

f.a.	PI	PS	PC	PE	SM
14:0	1.1 ± 0.9	0.9± 0.3	0.4±0.1	0.3±0.2	1.2±0.3
15:0	0.2 ± 0.1	0.2± 0.1	0.2±0.1	-	0.3±0.1
16:0	14.8 ± 3.4	16.6± 2.3	30.1±2.9	10.8±0.4	42.9± 3.3
16:1 (n-9)	0.4 ± 0.3	1.0±0.6	1.0±0.3	0.7±0.2	0.8±0.1
17:0	0.3 ± 0.1	0.3± 0.1	0.2±0.1	0.2± 0.1	0.7±0.1
18:0	25.7 ± 3.2	19.8±2.3	15.7±1.2	14.4±1.0	17.5±1.7
18:1c (n-9)	6.7 ± 1.6	15.9±1.3	14.0±0.8	9.5±0.4	7.2± 0.8
18:1t (n-9)	1.3 ± 0.3	3.7± 0.6	3.5±0.3	2.8± 0.2	1.4±0.6
18:2 (n-6)	4.2 ± 2.2	4.7±1.1	4.6±0.2	3.9±0.5	2.8±0.9
20:0	tr	tr	-	-	5.8±1.2
20:3 (n-6)	1.0 ± 0.5	1.4±0.3	1.2±0.3	0.5± 0.2	-
20:4 (n-6)	28.3 ±13.4	15.1±3.1	20.3 ±2.6	33.5±1.4	-
20:5 (n-3)	3.5 ± 1.7	2.5±0.6	1.4±0.4	2.2±1.2	-
22:0	-	-	-	-	5.2±0.9
22:4 (n-6)	1.1 ± 0.6	1.9±1.0	0.6±0.1	1.3±0.6	-
22:5 (n-3)	0.8 ± 0.3	1.6± 0.9	1.0±0.6	1.5± 0.8	-
22:5 (n-6)	1.4 ± 0.8	1.9± 0.1	1.2±0.4	4.9±0.1	-
22:6 (n-3)	9.5 ± 4.4	12.8± 3.1	4.7±1.4	13.5± 2.5	-
24:0	-	-	-	-	10.3±1.6
24:1 (n-9)	-	-	-	-	4.1±0.5
Total Saturates	42.1	37.7	46.6	25.7	83.8
Total Monosaturates	8.4	20.6	18.5	13.0	13.5
Total (n-6) Polyunsaturates	35.9	25.0	27.9	44.2	2.8
Total (n-3) Polyunsaturates	13.8	16.9	7.1	17.2	0
Total Polyunsaturates	49.7	41.9	35.0	61.4	2.8

Table 4.10 Fatty acid composition of phospholipids from the rat olfactory tissue.

Data is expressed as wt% and represent means ± standard deviation of 5-8 separate experiments. Rat olfactory turbinates weighed between 50-100 mg. The lipids were extracted with acidified chloroform-methanol 2:1 v/v and the phospholipids separated by thin layer chromatography, as detailed in Methodology. Individual spots were visualised under ultraviolet light, after spraying the TLC plates with ANSA (0.001% w/v), and eluted from the plates. Fatty acid methyl esters of individual phospholipids were prepared by esterification by BF_3-CH_3OH and analysed by GC/MS as described in Methodology. (See also Fig. 4.13)

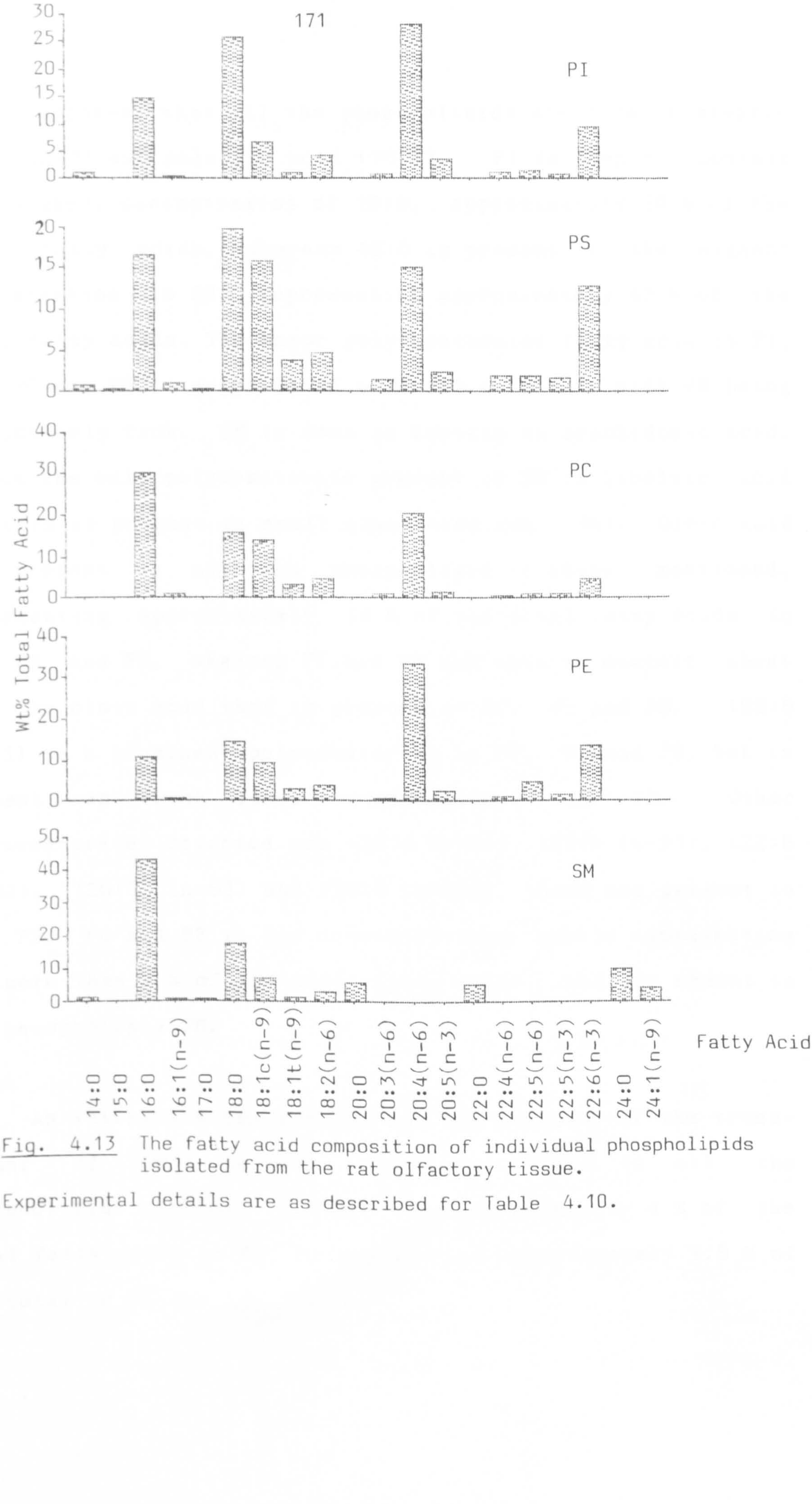


Fig. 4.13 The fatty acid composition of individual phospholipids isolated from the rat olfactory tissue.

Experimental details are as described for Table 4.10.

It is apparent that all the phospholipids are rich in stearic acid (18:0) and palmitic acid (16:0). PI is seen to contain the highest concentration of 18:0, approximately 26 % of the total fatty acids, whereas 16:0 is present in the highest concentration in SM, representing approximately 43 % of the total fatty acids. The major polyunsaturated fatty acid in PI, PS, PC and PE is arachidonic acid (20:4 [n-6]), with PE being particularly rich. SM is seen to contain no arachidonic acid. Infact the only polyunsaturate present in SM is linoleic acid and this is present in small quantities (ca. 3%). Oleic acid is present in all the phospholipid classes mentioned, representing approximately 14 % of the total fatty acids in PC, PE and PS, whereas PI and SM are seen to contain about half the oleic acid that is present in PC, PE and PS. (22:6 [n-3]) is a prominent polyunsaturate in PI, PS and PE, but is present in much lower concentrations in PC. Other polyunsaturates detected are (22:4 [n-6]), (22:5 [n-3]), (22:5 [n-6]), (20:3 [n-6]) and (20:5 [n-3]); these are present in PI, PS, PC and PE in low concentrations, mostly representing not more than 2 % of the total fatty acids, and are absent in the phospholipid SM.

An intriguing observation was the presence of the trans-isomer of (18:1 [n-9]). This was found in all the phospholipid classes, representing approximately 4 % of the total fatty acids in PS, PC and PE, and approximately 1.3 % of the total in PI and SM.

Trans-(18:1) isomers are usually present in much lower concentrations than those found in the olfactory tissue. For example, Rocquelin et al.[71] while studying the fatty acid composition of human heart phospholipids, found that despite the fact that the human subjects consumed large quantities of hydrogenated fats, low levels (ca. 0.4-1.2 % of total fatty acids) of trans-(18:1) were detected. Similarly, Heckers et al.[72] obtained mean values of 0.4 % of trans-(18:1) in total heart lipids and Ohlrogge et al.[73] reported values of approximately 0.7 % of trans-(18:1) in heart phospholipid classes.

Overall, SM is observed to be different from all the phospholipid classes described, being highly saturated and containing polyunsaturates representing only 2.8 % of the total fatty acids. PE differs from PC and PS in having almost twice as much polyunsaturated fatty acids. The total amount of polyunsaturates in PI is approximately 50 % of the total fatty acids. PI, PS, PC and PE all contain higher concentrations of the [n-6] polyunsaturated fatty acids than the [n-3] polyunsaturated moieties which are present in much lower concentrations.

An attempt was also made to identify and measure the fatty acid concentrations in PIP, PIP_2 and PA of the olfactory tissue. This proved difficult, mainly due to these phospholipids representing only a small fraction of the total phospholipids. To overcome this limitation, the experiment

was performed by loading all the material from one animal on to several TLC plates and pooling the resulting separated phospholipids in the hope that enough material was present for the analysis of the fatty acids. GC/MS, however, was unable to detect the presence of any fatty acids. This experiment thus needs to be repeated with modifications. Perhaps, material from several animals need to be pooled for a single experiment.

4.4 CONCLUSION

The physical properties of the membrane are determined by the individual phospholipids therein, the fatty acid composition of these phospholipids, and their interaction with cholesterol and proteins which may be enzymes or part of the cytoskeletal material. This study attempted to investigate the role of phospholipids and their fatty acids in olfaction. The studies were preceded by investigating the type and concentration of lipid within the olfactory epithelium followed by studies on lipid metabolism.

The olfactory epithelium is found to contain high concentrations of phospholipids,ca. 80% of total lipid, the remaining 20% consisting of mainly neutral lipids. These levels are comparable to levels found in various other tissues (see Table 4.3). For example, Rabinowitz et al.[74], studying the lipid composition of steer tongue epithelium reported phospholipid levels of approximately 60% Of total

lipid. Similarly, the pig-liver lipid extract has been shown to consist of approximately 70% phospholipids and 30% neutral lipids[76]. Norton et al.[76], have investigated the lipid composition of isolated brain cells and axons. They show total phospholipid concentration in neurons, glia and the whole brain to be in the range of 65-86 % of the lipid weight. The lipid composition of the olfactory tissue can thus be said to be not very different from other tissues. If olfactory reception is mediated in part by plasma membrane lipids, then, at the levels of the phospholipid analysis carried out in this study, no unique distribution of membrane lipids is present in the olfactory tissue.

Membrane phospholipids are known to participate in cellular transductive events[77] and are generally considered to impart fluidity and stabilising properties on the membrane. They are thus integral structures in any membrane which maintains receptor elements. Over the past decade there has been a rapid accumulation of information concerning the role of receptor-stimulated PA and PI metabolism in the control of cellular activities. Nevertheless, until recently, little attention had been paid to the phosphorylated derivatives of PI, PIP and PIP_2. The polyphosphoinositides appear to be ubiquitous components of eukaryotic cells, although they represent only a minor proportion of total cell phospholipid[4]. Past neglect of the polyphosphoinositides may partly be explained by difficulties encountered in the study of such quantitatively minor phospholipids. Also, because of the

highly polar nature of these lipids, conditions of acidity or high ionic strength are necessary for their extraction and special thin layer chromatographic procedures are required for their separation. However, there is now much evidence to suggest that PIP and PIP_2 function in the transduction of signals across the cell membrane (see introduction, 4.1).

The olfactory tissue is found to be rich in inositol phospholipids including polyphosphoinositides. PI comprises about 10% and PIP and PIP_2 each represent approximately 2.5% and 1.7% of total phospholipid, respectively. In human erythrocytes, a tissue regarded as a rich source of polyphosphoinositides, PIP and PIP_2 together account for 1% of the total phospholipids[4]. Liver cells, where the plasma accounts for a smaller proportion of total cellular membrane, contain much lower levels of polyphosphoinositides[78]. In this study ^{32}P orthophosphate is readily incorporated into PA, PI, PIP and PIP_2 of the rat olfactory tissue incubated in Ringers and no other additions. Thus the inositol lipids are metabolically active in the unstimulated state. The much higher levels of radioactivity incorporated in PIP and PIP_2 than in PA and PI are consistent with rapid dephosphorylation of PIP and PIP_2 by phosphomonoesterases and rapid rephosphorylation of the resulting products by kinases, as is thought to occur in other systems[82].

Levels of ^{32}P orthophosphate incorporation into the polyphosphoinositides appear to decline in the presence of an

odour. Although these results appear to be consistent with similar experiments carried out on rat hepatocytes by Creba et al.[65] and Thomas et al.[66], much work is required in order to substantiate the above findings. Odour stimulation of the intact olfactory tissue provides conflicting data to the above study. Recently, King et al.[79] in a study on human erythrocytes suggest possible metabolic pooling of the membrane phospholipids PA, PIP and PIP_2. Their findings indicate that there is metabolic comparmentalisation of PA, PIP and PIP_2 within the cells and that only a fraction of these lipids undergo metabolic turnover. If this was also true for olfactory phospholipids then the above approach used in this study to measure changes in incorporation of radioactivity may not be sensitive enough to detect any changes in PI turnover (presumably in the cilia) that may occur in the presence of an odour.

An interest in the possible roles of phospholipids and fatty acids in olfaction prompted an analysis of the fatty acid composition of the olfactory phospholipids. The polyunsaturated fatty acids of the olfactory phospholipids are dominated by (n-6) rather than (n-3) moieties. This observation is based mainly on the analyses of the major phospholipids in olfactory tissue. Fatty acid analysis of the minor polyphosphoinositides proved difficult mainly due to these phospholipids representing very small concentrations of the total. Generally, phospholipids of terrestrial mammals are characteristically rich in (n-6)

polyunsaturated fatty acids[66]. This appears to be true for the olfactory tissue as well. Docosahexaenoate (22:6 [n-3]) is known to be a major acyl moiety of PC, PE, and PS in excitatory tissues such as the brain cortex, retina and heart[60]. In the olfactory tissue this polyunsaturate is seen to be prominent in the phospholipids PI, PE and PS; it is less distinctive in PC. Arachidonic acid (20:4 [n-6]) is the major polyunsaturated fatty acid in PC, PE, PS and PI of the olfactory epithelium. This finding is consistent with other studies on terrestrial mammals[67]. It can thus be concluded that the fatty acid profile of the rat olfactory phospholipids is not unique to the olfactory tissue. The presence of elaidic acid (trans 18:1 [n-9]), however, is of interest since it appears to be present in much higher concentrations than is observed in other tissues[71]. Whether this difference in levels is of significant importance to olfaction needs yet to be established.

In the recent years there has been a great surge of interest regarding the role of phospholipids and their fatty acids in transduction mechanisms. It is particularly interesting that PI, now confirmed to be an intermediate in the inositol phospholipid cycle, also serves as a source of arachidonic acid (20:4 [n-6]) a precursor of prostaglandins E_2 and F_2 . Blomstrand et al.[70] in their recent study on rat retina have shown that there is a pronounced change in the arachidonic content in PI and a decreased amplitude of the corresponding a-wave of the

electro-retinogram. They therefore suggest that arachidonic acid of the PI has an important function in the visual phototransduction process. These same workers have also demonstrated that non-human primates deprived of dietary (n-3) fatty acids during gestation and infancy showed impairment of visual acuity. Data in the literature from several sources suggest that (n-3) fatty acids may have a specific function in the photoreceptor membranes of the retina. Since olfaction seems to bear a striking molecular similarity to visual reception (see chapter 2), it would be of interest to investigate the effect of diet on the fatty acid composition of rat olfactory phospholipids and the resulting EOG.

Phospholipids containing docosahexaenoate (22:6 [n-3]) are thought to have specific molecular roles in active calcium transport in sarcoplasmic reticulum and retinal photoreceptor discs[81]. Olfactory phospholipids PI, PS and PE , in this study are found to contain (22:6[n-3]) in prominent amounts. It would therefore be interesting to establish the possible role of this polyunsaturate in olfaction.

The lipid composition of naturally occurring membranes is generally quite heterogeneous. At the present time, the relationship between specific lipid composition and membrane structure and catalyic activity is not well understood. Furthermore, little is known about regulative mechanisms governing membrane composition. A body of evidence

indicates that hydrolysis of inositol lipids is involved in the control of a variety of cellular processes including phototransduction[14]. Polyunsaturated fatty acids determine the fluidity of the membrane and appear to play an important role in excitatory tissues[70,81]. It is thus hoped that this study will stimulate the necessary research to elucidate the metabolic roles of these lipids in the olfactory tissue.

REFERENCES

1. Hokin, M.R. and Hokin, L.E. (1953) J. Biol. Chem., 203, 967-977.

2. Esko, J.D. and Raetz, C.R.H. (1980) J. Biol. Chem., 255, 4474.

3. Michell, R.H. (1975) Biochem. Biophys. Acta, 415, 81-147.

4. Downes, C. and Michell, R.H. (1982) Cell Calcium, 3, 467-502.

5. Paulus, H. and Kennedy, E.P. (1960) J. Biol. Chem., 235, 1301.

6. Grado, C. and Ballon, C.E. (1961) J. Biol. Chem., 236, 54.

7. Nishizuka, Y. (1986) Science, 233, 305.

8. Abdel-Latif, A.A., Akhtar, R.A., Hawthorne, J.N. (1977) J. Biochem., 162, 61.

9. Putney, J.W., Weiss, S.J., Van de Walle, C.M. and Haddas, R.A. (1980) Nature, 284, 345-347.

10. Nishizuka, Y. (1984) Nature, 308, 693-697.

11. Kraft, A.S. and Anderson, W.B. (1983) Nature, 301, 621-623.

12. Castagna, M. Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem., 257, 7847-7851.

13. Storey, D.J., Shears, S.B., Kirk, C.J. and Michell, R.H. (1984) Nature, 312, 374-376.

14. Berridge, M.J. and Irvine, R.F. (1984) Nature, 312, 315-321.

15. Berridge, M.J., Downes, C.P. and Hanley, M.R. (1982) J. Biochem, 206, 587-595.

16. Wilson, D.B., Connolly, T.M>, Bross, T.E., Majerus, P.W., Sherman, W.R., Tyler, A.N., Rubin, L.J. and Brown, J.E. (1985) J. Biol. Chem., 260, 13496-13501.

17. Wilson, D.B., Neufeld, E.J. and Majerus, P.W. (1985) J. Biol. Chem., 260, 1046-1051.

18. Batty, I.R., Nahorski, S.R. and Irvine, R.F. (1985) J. Biochem., 232, 211-215.

19. Heslop, J.P., Irvine, R.F., Tashjian, A.H. and Berridge, M.J. (1985) J. Exp. Biol., 119, 395-401.

20. Irvine, R.F., Anggord, E.E., Letcher, A.J. and Downes, C.P. (1985) J. Biochem., 229, 505-511.

21. Majerus, P.W. (1983) J. Clin. Invest., 72, 1521.

22. Bell R.L., Kennerly, D.A., Stanford, N. and Majerus, P.W. (1979) Proc. Natl. Acad. Sci., 76, 3238.

23. Mckean, M.L., Smith, J.B. and Silver, M.J. (1981) J. Biol. Chem., 256, 1522.

24. Hofmann, S.L. and Majerus, P.W. (1982) J. Biol. Chem., 257, 6461.

25. Low, M.G., Carroll, R.C. and Weglicki, W.B. (1984) J. Biochem., 221, 813.

26. Wilson, D.B., Bross, T.E., Hofmann, S.L., Majerus, P.W. (1984) J. Biol. Chem., 259, 11718.

27. Hofmann, S.L. (1983) Ph.D. Thesis, Washington University, St. Louis, USA.

28. Billah, M.M. and Lapetina, E.G. (1983) Proc. Natl. Acad. Sci., 80, 965.

29. Feinstein, M.B., Egan, J.J., Sha'afi, R.I. and White, J. (1983) Biochem. Biophys. Res. Commun., 113, 598.

30. Gomperts, B.D. (1980) Nature, 284, 17.

31. Litosch, I., Wallis, C. and Fain, J.N. (1985) J. Biol. Chem., 260, 5464.

32. Cockroft, S. and Gomperts, B.D. (1985) Nature, 314, 534-537.

33. Wallace, M. and Fain, J.N. (1985) J. Biol. Chem., 260, 9527.

34. Gonzales, R.A. and Crews, F.T. (1985) J. Biochem., 232,799.

35. Straub, R.E. and Gershengorn, M.C. (1986) J. Biol. Chem., 261, 2712.

36. Wilson, D.B., Bross, T.E., Sherman, W.R., Berger, R.A., Majerus, P.W. (1985) Proc. Natl. Acad. Sci., 82, 4013.

37. Wilson, D.B., Bross, T.E., Sherman, W.R., Berger, R.A., Majerus, P.W. (1985) J. Biol. Chem., 260, 13496.

38. Brass, L.F. and Joseph, S.K. (1985) J. Biol. Chem., 260, 15172.

39. Habenicht, A.J.R. (1985) J. Biol. Chem., 256, 12329.

40. Pace, U., Hanski, E., Salomon, Y. and Lancet, D. (1985) Nature, 316, 255-258.

41. Sklar, P.B., Anholt, R.R.H. and Snyder, S.H. (1986) J. Biol. Chem., 261, 15538-15543.

42. Huque, T. and Bruch, R.C. (1986) Biophys. Biochem. Res. Commun., 137, 36-42.

43. Anholt, R.R.H., Mumby, S.M., Stoffers, D.A., Girard, P.R., Kuo, J.F. and Snyder, S.H. (1987) Biochem., 26, 788-795.

44. Cagan, R.H. (1981) "Biochemistry of Taste and Olfaction" (Cagan, R.H. and Kare, M.R., eds.) Academic Press, New York, pp. 175-203.

45. Koyama, N. and Kurihara, K. (1972) Nature, 236, 402-404.

46. Cherry, R.L., Dodd, G.H. and Chapman, D. (1970) Biochim. Biophys. Acta, 211, 409-416.

47. Fesenko, E.E., Novoselov, V.I., Pervukhim, G.Ya. and Fesenko, N.K. (1977) Biochim. Biophys. Acta, 466, 347-356.

48. Nomura, T. and Kurihara, K. (1987) Biochem., 26, 6135-6145.

49. Simpson, C.M.F. and Sargent, J.R. (1985) Comp. Biochem. Physiol., 82b, 781-786.

50. Folch, J., Lees, M. and Stanley, G.H.S. (1957) J. Biol. Chem., 226, 497-509.

51. Parsons, J.G. and Patton, S. (1967) J. Lipid Res., 8, 696-698.

52. Schacht, J. (1981) Meth. Enzym., 72, 626-631.

53. Jolles, J., Zwiers, H., Dekker, A., Wirtz K.W.A. and Gispen, W.H. (1981) Biochem. J., 194, 283-291.

54. Bartlett, G.R. (1959) J. Biol. Chem., 234, 466.

55. Pollet, S., Ermidou, S., Saux, F.Le., Mongue, M. and Baumann, N. (1978) J. Lipid. Res., 19, 916-921.

56. Drenthe, E.H.S. and Daemen, F.J.M. (1982) Meth. Enzymol., 81, H, 320-329.

57. Bell, M.V., Simpson, C.M.F. and Sargent, J.R. (1983) Lipids, 18, 10, 720-726.

58. Sugiura, Y. (1981) Biochem. Biophys. Acta, 641, 148-159.

59. Folch, J. (1949) J. Biol. Chem., 177, 497-504; Ibid. 177, 505-519.

60. Le Baron, F.N. and Folch, J. (1956) J. Neurochem., 1, 101-108.

61. Hawthorne, J.N. and Pickard, M.R. (1979) J. Neurochem., 32, 5-14.

62. Irvine, R.F., Letcher, A.J. and Dawson, R.M.C. (1984) J. Biochem., 218, 117-185.

63. Dawson, R.M.C. (1979) "Data for Biochemical Research", 2nd Edition, eds. Dawson, R.M.C., Elliot, D.C., Elliot, W.H., and Jones, K.M. (Clarendon Press, Oxford).

64. Shirley, S., Polak, E., and Dodd, G.H., (1983) Eur. J. Biochem., 132, 485-494.

65. Creba, J.A., Bownes, C.P., Hawkins, P.T., Brewster, G., Michell, R.H. and Kirk, C.J. (1983) J. Biochem., 212, 733-747.

66. Thomas, A.P., Marks, J.S., Coll, K.E. and Williamson, J.R. (1983) J. Biol. Chem., 258, 9, 5716-5725.

67. Gurr, M.I. and James, A.T. (1980) "Lipid Biochemistry: An Introduction", 3rd Edition (Chapman and Hall, London).

68. Holub, B.J. and Kuksis, A. (1978) Adv. Lipid Res., 16, 1-125.

69. Marshall, P.J., Boatman, D.E. and Hokin, L.E. (1981) J. Biol. Chem., 256, 844-847.

70. Blomstrand, R., Sisfontes, L. and Ingemansson, S. (1986) Bioscience Reports, 6, 543-556.

71. Rocquelin, G., Guenot, L., Justrabo, A., Grynberg, A. and David, M. (1985) Mol. Cell. Cardiol., 17, 769-773.

72. Heckers, H., Korner, M., Tuschen, T.W.L. and Melcher, F.W. (1977) Atherosclerosis, 28, 389-398.

73. Ohlrogge, J.B., Emken, E.A. and Gulley, R.M. (1981) J. Lipid Res., 22, 955-960.

74. Rabinowitz, J.L., Brand, J.G. and Bayley, D.C. (1982) Lipids, 17, 12, 950-955.

75. Shand, J.H. and Noble, R.C. (1980) Analyt. Biochem., 101, 427-434.

76. Norton, W.T., Abe, T., Poduslo, S.E. and De Vries, G.H., (1975) J. Neuroscience Res., 1, 57-75.

77. Michell, R.H. and Bone, E.A. (1985) Symposia Medica Hoechst., 19, 399-411.

78. Michell, R.H., Hawthorne, J.N., Coleman, R. and Karnovsky, M.L. (1970) Biochim. Biophys. Acta, 210, 86-91.

79. King, C.E., Stephens, L.R. Hawkins, P.T., Guy, G.R. and Michell, R.H. (1987) J. Biochem., 244, 209-217.

80. Infante, J.P. (1985) J. Theor. Biol., 116, 65-88.

81. Infante, J.P. (1987) Mol. Cell. Biochem., 74, 111-116.

82. Gunstone, F.D., Harwood, J.L., Padley, F.B., eds., (1986) "The Lipid Handbook" (Chapman and Hall, London).

CHAPTER 5

GENERAL CONCLUSIONS

The biochemical properties of putative olfactory receptor molecules have remained largely unknown. No information concerning the structure and function of an olfactory receptor or enzymes that may be coupled to it is available.

Measurement of odorant-stimulated adenylate cyclase in olfactory cilia provides the first in-vitro assay for olfactory reactivity and could complement electrophysiological recordings in the study of odorant recognition. The olfactory adenylate cyclase should be a powerful tool for the study of the olfactory receptors in-vitro, providing a monitor for the solubilisation and purification of the receptors.

Most of the criteria necessary for establishing the role of cyclic AMP as a second messenger in olfactory reception have been fulfilled. However, the 1st Sutherland criterion (i.e. odorants should alter the intracellular levels of cyclic AMP in the receptor cells), has yet to be verified. Attempts in this study to stimulate intracellular levels of cyclic AMP proved difficult, partly due to the laborious and time-cosuming procedures used for the fixation and extraction. The assay employed for this investigation may not be sensitive enough to detect any changes in the cyclic AMP levels.

It is found that not all odorants activate the olfactory adenylate cyclase. This may suggest the existence of other transduction mechanisms that may be involved in olfaction. Membrane lipids are known to participate in cellular transduction events. It is considered possible that the lipids of the plasma membrane may be partially responsible for the recognition steps in certain olfactory responses. There has, however, been a lack of basic knowledge of the type, concentration and metabolism of lipids within the olfactory epithelium. This study is the first attempt to investigate the lipid profile of the olfactory epithelium. The olfactory epithelium is found to be a rich source of phospholipids. No unique distribution of phospholipids is observed. Metabolic studies of the lipids show phospholipids to be metabolically active in the unstimulated state. The effect of the presence of an odour on the inositol lipid turnover has not been clearly established.

The fatty acid profile of the rat olfactory phospholipids is also not unique to the olfactory tissue. The elaidic acid, (trans-18:1[n-9]), is found to be present in higher concentrations compared to other tissues. Whether this difference in levels is of significant importance to olfaction needs to be established.

Polyunsaturated fatty acids determine the fluidity of the membrane and appear to play an important role in excitatory tissues. Factors such as diet are thought to

affect the fatty acid compositions. It would therefore be of interest to investigate the possible roles of these polyunsaturates in olfaction. For instance, the effect of diet on the fatty acid composition of olfactory phospholipids and the resulting EOG responses could be investigated.

The lipid composition of naturally occurring membranes is generally quite heterogeneous. At present, the relationship between specific lipid composition and membrane structure and catalytic activity is not well understood. It is thus hoped that this study will stimulate the necessary research to elucidate the metabolic role of these lipids in the olfactory tissue.